Function and regulation of the multidrug resistance-associated protein 1 during inflammatory bowel disease and liver regeneration

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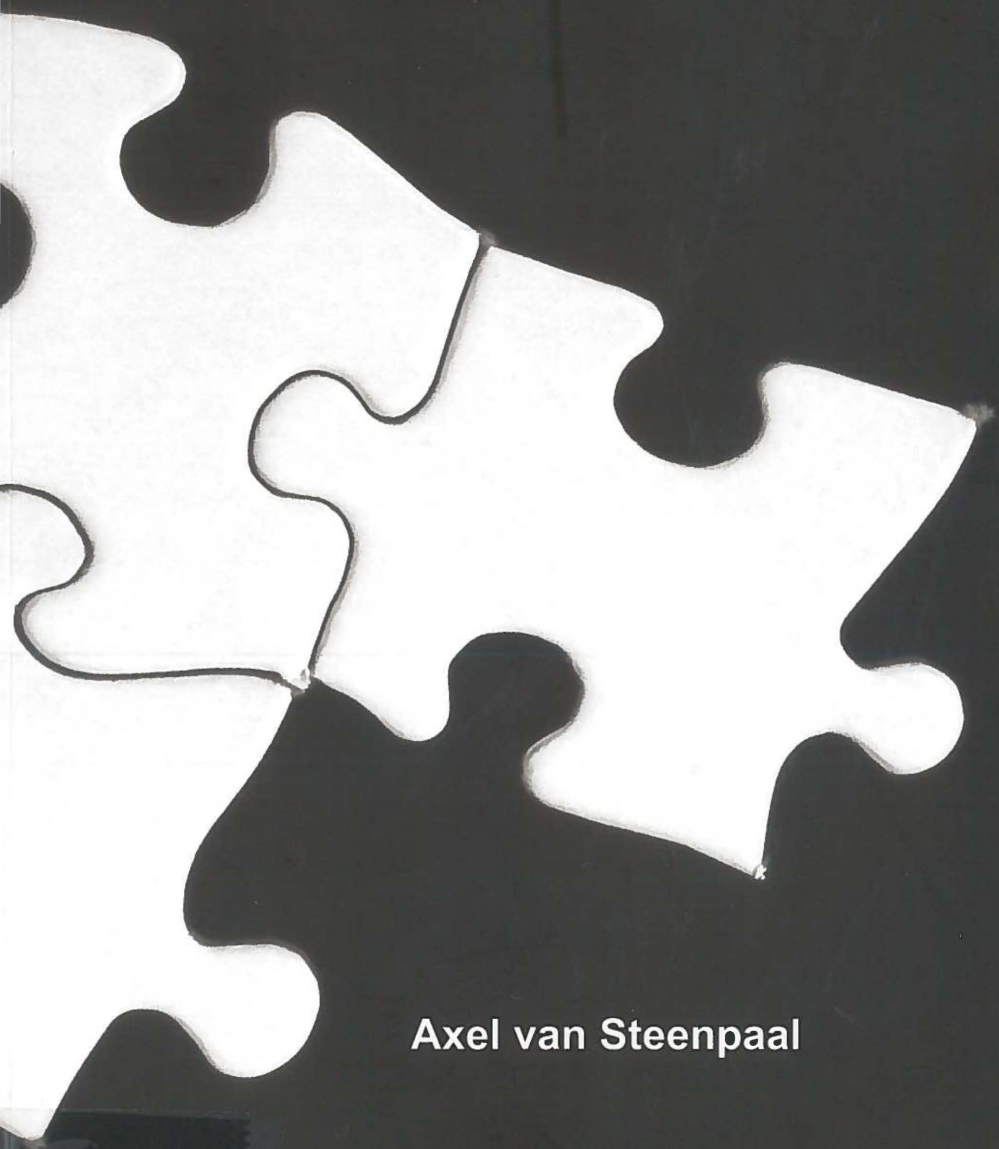

Axel van Steenpaal

Function and regulation of the multidrug resistance-associated protein 1 during inflammatory bowel disease and liver regeneration

ISBN: 978-94-610-8129-2

Cover and page design: Axel van Steenpaal
Photograph obtained from http://nurturingfaith.files.wordpress.com/
Printed by: Gildeprint drukkerijen, Enschede

Stellingen behorend bij het proefschrift:
"Function and regulation of the multidrug resistance associated protein 1 in inflammatory bowel disease and liver regeneration"

1. Expressie van MRP1 in humaan darmepitheel wordt, in tegenstelling tot in muizen, niet gereguleerd door PPAR-α

2. Hoewel de invloed van glutathion efflux op de functie van MRP1 tijdens anti-FAS geïnduceerde celdood in Jurkat cellen omstreden is, kan worden vastgesteld dat de intracellulaire glutathion concentratie door MRP1 niet verandert.

3. Tijdens leverregeneratie in muizen vertaalt een verhoging van de expressie van stamcelmarker alpha fetoproteine zich niet in zichtbare ductulaire reacties

4. Vanwege de bewezen beschermende werking van MRP1 in orgaan specifieke stamcellen biedt modulatie van MRP1 expressie interessante mogelijkheden voor de regeneratieve geneeskunde

5. Het huidige aantal proefdieren dat gebruikt wordt in het wetenschappelijk onderzoek kan drastisch teruggebracht worden door efficiëntere samenwerking tussen verschillende onderzoeksgroepen

6. Het is niet de computer die langzamer wordt, maar de onrust die groeit bij de gebruiker ervan

7. Het verhogen van de marktwerking in de gezondheidszorg en het, mede hierdoor, ontstane preferentiebeleid voor geneesmiddelenvergoeding kan nooit leiden tot een optimale patiëntenzorg

8. In kip-kerrie salade hoort geen ananas, anders had het wel kip-kerrie-ananas salade geheten

9. De beoordeling van andermans werk is meestal gebaseerd op de hoeveelheid kritiek die erop gegeven kan worden in plaats van de innovatieve kennis die het bevat

10. De kunst van het bereiken van succes is weten wanneer je moet stoppen

Axel van Steenpaal

cmb.

RIJKSUNIVERSITEIT GRONINGEN

Function and regulation of the multidrug resistance-associated protein 1 during inflammatory bowel disease and liver regeneration

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 2 februari 2011
om 13:15 uur

door

Axel van Steenpaal

geboren op 2 maart 1979
te Emmen

Promotores:	Prof. dr. K.N. Faber Prof. dr. A.J. Moshage
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CHAPTER 1

Introduction

A. van Steenpaal

Multidrug resistance is a phenomenon first observed when drugs were tested to treat cancer. The cancer cells developed resistance against the tested drug and at the same time also against other, structurally-unrelated drugs. This catalyzed the search for the molecular mechanism, resulting in the identification of a substrate transport protein that is able to export a wide variety of drugs out of cancer cells, the P-glycoprotein or multidrug resistance protein 1 (MDR1). Soon after, similar solute transporters were identified that showed comparable characteristics and were called multidrug resistance-associated proteins (MRPs). MDR1 and MRPs are also expressed in healthy tissue, but their physiological role was unclear for a long time. MDR1 and MRPs are particularly present in barrier forming cells, such as in the liver (blood-bile), intestine (blood-gut lumen) and brain (blood-brain). This thesis describes the function and regulation of the Multidrug resistance-associated protein 1 in intestinal and liver diseases. First, an overview will be given of the function and cellular architecture of the intestine and liver, followed by a description of relevant diseases of these organs, e.g. inflammatory bowel diseases and liver regeneration. Next, the superfamily of ATP-binding cassette (ABC)-transporters will be introduced with specific emphasis on MRP1, a member of the ABC subclass C. Finally, the present knowledge of the function and regulation of MRP1 during pathological conditions will be summarized, followed by the scope of this thesis.

1. The intestine

1.1 Intestinal function and architecture

The intestine performs the important task of uptake of nutrients from ingested foods. It consists of several morphologically different sections, which can be roughly divided into the small intestine and the large intestine (colon). In the small intestine food is mixed with fat-solubilizing bile and digestive enzymes that make nutrients accessible for absorption. In the colon, electrolytes and water are reabsorbed from the bowel content. To maximize the absorption surface, both the small and large intestinal surface is heavily folded forming crypts and villi.

The intestinal wall can be roughly divided into 4 sections; the mucosa, submucosa, muscularis externa and serosa (Figure 1A). The serosa is the outer, protective layer of connective tissue, the muscularis externa is the layer of smooth muscle that controls the bowel movement (both constricting as well as longitudinal). The submucosa is a layer of connective tissue that contains nerves that connect to the muscularis mucosa and a matrix of blood vessels that run in to the mucosa. The most inner layer and first barrier for the content of the gut lumen is the mucosa.

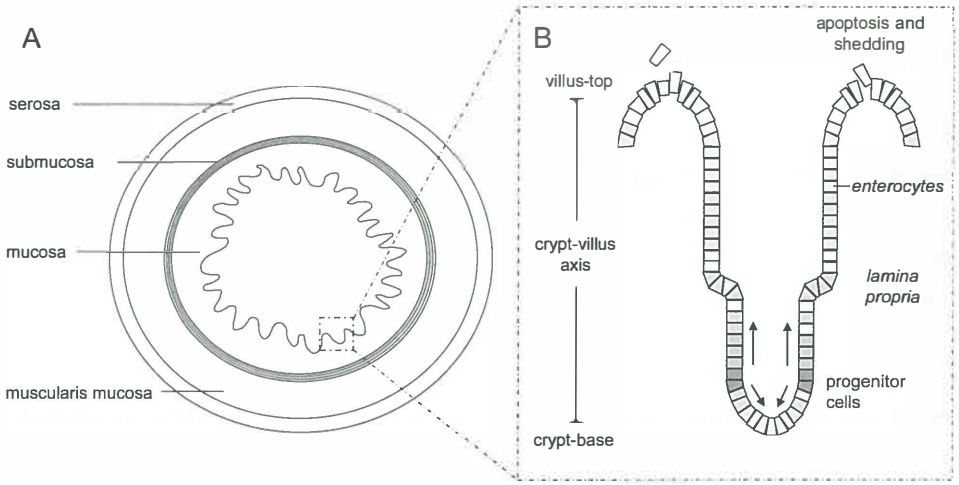

***Figure 1**. Architecture and cell types of the intestine. (A) Schematic representation of a cross section of the intestinal wall. Four (4) functional layers of tissue can be discriminated. The outer layer is the serosa, which is a protective layer of connective tissue. Next, the muscularis mucosa is a layer of muscle, responsible for bowel movement. The submucosa contains blood vessels and elastic fiber with collagen that maintains the shape of the intestine. Moreover, it contains nerves that connect to the muscularis mucosa. The most inner layer is the mucosa. In this layer, uptake of nutrients takes place. (B) Schematic representation of a crypt and two villi in the mucosa of the intestine. The intestinal progenitor cell compartment is located at the base of the crypt. Intestinal progenitor cells proliferate continuously both towards the top of the villus as well as the base of the crypt. The newly formed cells that migrate into the villus differentiate into mature intestinal epithelial cells.*

The mucosa consists of another 3 sublayers, the muscularis mucosa, the lamina propria and the mucosa (or epithelium). The muscularis mucosa is a thin layer of smooth muscle that maintains the folded surface area in the intestine. Above the muscularis mucosa, the lamina propria is a layer of connective tissue that contains many lymph nodes harboring immune cells for protection against pathogens residing in the intestine. Finally, the layer that is in direct contact with the bowel content is the epithelium. The epithelium contains several different cell types with highly specialized functions; e.g. the production of mucin (by Goblet cells), secretion of antibacterial enzymes (by Paneth cells), secretion of hormones (enteroendocrine cells) or the absorption of nutrients (enterocytes) (1). On top of the epithelium, a non-cellular barrier is present in the form of a thick mucin layer, completing the protective layer of the intestinal wall.

The intestine contains billions of resident bacteria, which aid in the digestion of food. The intestinal mucosa prevents bacteria from penetrating into the underlying tissue or the circulation by forming a tight barrier of epithelial cells. In addition, the lamina propria provides large amounts of immune cells to remove bacteria that do penetrate the mucosa. To maintain the epithelial cell barrier, the intestine is continuously

renewing the epithelial cell layer by proliferation and differentiation of intestinal stem cells, located in the base of the crypts (Figure 1B) (reviewed in (2),(3)). Although their exact location in the crypt is still a matter of debate (reviewed in (4)) it is known that these stem cells differentiate into mature epithelial cells as they move up towards the villus tip. There, they are lost by controlled cell death (apoptosis) and/or shedding. In this way, the intestinal epithelium is completely renewed every 2-5 days (5). The intestinal progenitor cells can differentiate into all different epithelial cell types e.g. enterocytes, Paneth cells, Goblet cells and enteroendocrine cells.

1.2 Inflammatory bowel disease

One of the most prevalent intestinal diseases in the Western world is inflammatory bowel disease (IBD), with an estimated 2.2 million patients in Europe and 1-1.4 million patients in the US. IBD include Crohn's disease (CD) and ulcerative colitis (UC) (6). Both diseases are characterized by a dysregulated immune response to the endogenous microflora, resulting in continuous inflammation and ulceration of the intestinal mucosa (7). Characteristic symptoms are abdominal pain, (bloody) diarrhea and loss of appetite. Although CD and UC share common symptoms, they also show highly specific characteristics. Inflammation can be found throughout the entire gastrointestinal tract (from mouth to anus) in CD, but generally starts in the terminal ileum and spreads into the colon. In contrast, inflammation in UC starts from the anus, from which it spreads into the colon. The ileum, however, is never affected in UC. Furthermore, CD displays a "patchy" pattern of ulceration, which can penetrate into all layers of the intestinal wall, while UC shows a continuous inflammation which is restricted to the outer layer of the colon mucosa. It is evident that both CD and UC are caused by abnormal immune responses against the endogenous gut flora. This is indicated by the fact that animals kept in germ free conditions do not develop intestinal inflammation (8) and that antibiotics and probiotics can decrease the inflammation in IBD patients (reviewed in (9)).

A genetic predisposition has been demonstrated for both CD and UC, but this appears more pronounced in CD. One of the first genes in which mutations were found to be associated with CD was located in the previously discovered susceptibility region (IBD1) of chromosome 16 (10), CARD15/NOD2 (caspase recruitment domain family, member 15 / nucleotide-binding oligomerization domain containing 2) (11). NOD2 encodes a protein that senses the bacterial wall product muramyl dipeptide, suggesting that impaired sensing of penetrated bacteria into the intestinal mucosa is involved in the development of CD. Next, OCTN, encoding an organic cation transporter, and DLG5, encoding a protein involved in epithelial integrity,

were identified to be associated with CD (both OCTN and GLD5) (12,13) and UC (GLD5) (13). Therefore, decreased epithelial integrity seems to be involved in the development of both CD and UC.

The past 2 years have revealed an enormous network of genes that are associated with CD and UC. This is the result of genome-wide association studies (GWASs) that analyzed high numbers of genomic single nucleotide polymorphisms (SNPs) in large cohorts of IBD patients compared to controls (reviewed in (14,15). Currently, approximately 80 alleles have been shown to be associated with CD, while the number is now approximately 30 for UC. These GWAS allow the detection of specific cellular mechanism that predispose for the development of IBD and much research is now being performed in analyzing the functions of these genes and the relevance for intestinal inflammation. However, it should be noted that IBD develops as a result of genetic predisposition and environmental factors, where the latter appears to be the most relevant for the development of disease.

2. The liver

2.1 Liver function

The liver is one of the largest organs in the human body. It is an omnipotent organ that serves several important functions. Toxic compounds are metabolized in the liver, so that they can be excreted from the body. This occurs mainly by conjugation of these compounds to glutathione, glucuronide or sulphate to produce more hydrophilic metabolites, which can be excreted via the kidneys into urine. In addition, toxic compounds are excreted via the bile into the intestine. The liver produces bile salts that are required for the excretion of hydrophobic compounds from the liver, as well as for the uptake of essential lipid-soluble fats and vitamins from the intestine. Other important functions of the liver are sensing and elimination of pathogens, regulation of blood glucose levels and storage of vitamins, amino acids and glycogen.

2.2 Liver architecture

Before returning to the heart, blood from (nearly) all organs is transported to the liver via the upper or lower portal vein. The venous blood carries nutrients from the intestine as well as waste products from other organs. Oxygenated blood is delivered to the liver via the hepatic artery. Venous and arterial blood is channeled into small blood vessels (sinusoids), which are lined by endothelial cells (Figure 2A). Beneath the endothelial cell layer lies a string of the functional and most dominant cell type in the liver, the hepatocytes. Here, compounds are taken up from the blood through the basolateral membrane (facing the blood), metabolized or stored and

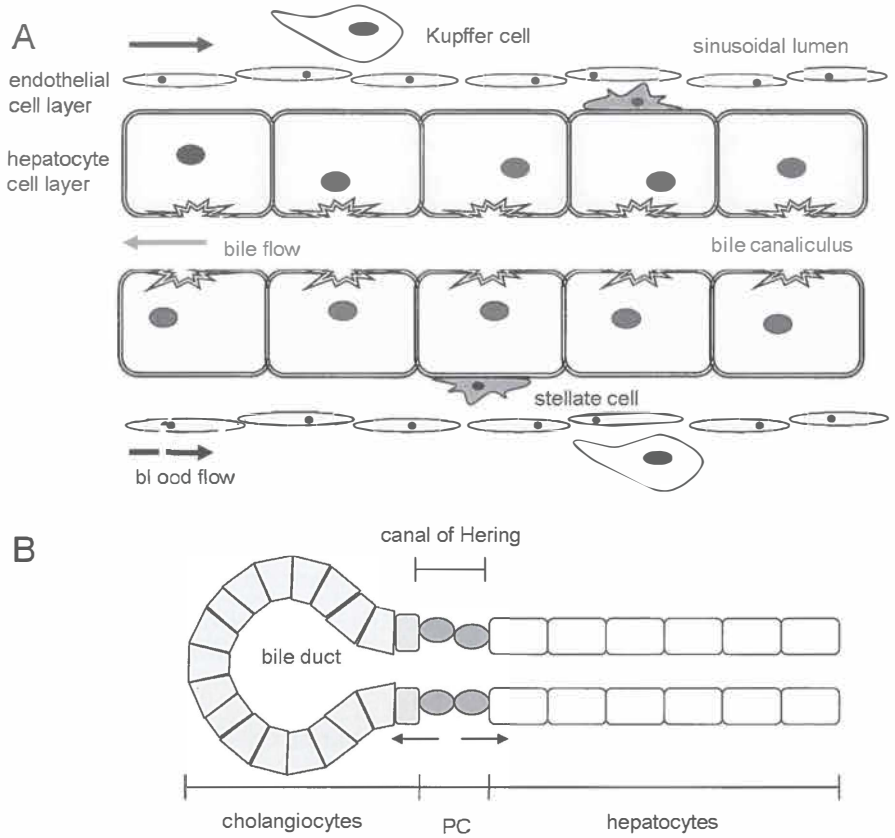

***Figure 2**: Cell types in the liver. (A) Sinusoids are lined by endothelial cells. Kupffer cells (liver-specific macrophages) are located in the sinusoids. Stellate cells reside in the space of Disse that separates the endothelial cells and hepatocytes. Strings of hepatocytes are lined between the portal vein and central vein. Hepatocytes transport bile salts, cholesterol and waste products into the bile canaliculi. Bile then moves to the gall bladder via the bile ducts. (B) The progenitor cell compartment in the liver is located in the canals of Hering (the interphase of hepatocytes and cholangiocytes).*

excreted again when necessary. Hepatocytes export bile salts, phospholipids and cholesterol via the apical (or canalicular) membrane into the bile canaliculi, through which these bile components flow via the bile ducts into the gallbladder for storage. Upon food intake, bile is released from the gallbladder into the gut. It mixes with the bowel content, thereby solubilizing the fat-soluble nutrients. At the end of the ileum (small bowel) the bile acids and nutrients are taken up by enterocytes returned to the liver via the portal vein. Bile salts are re-used in this so-called enterohepatic circulation.

2.3 Liver diseases

Liver diseases are very common and can have numerous causes, such as (viral) infections like hepatitis B or C, autoimmune disorders, metabolic syndrome,

fungal toxins, drug and alcohol abuse. Liver diseases can be subdivided in acute and chronic forms. Acute liver disease, for example resulting from de novo viral infections or drug-intoxication, lead to a very rapid loss of functional liver tissue (particularly hepatocytes) and requires curative treatment within days to prevent liver failure. Chronic liver diseases develop over many years to decades. This is typically seen in patients with persistent viral infection, autoimmune disorders, metabolic syndrome and drug/alcohol abuse. Functional liver tissue is replaced by activated and proliferating hepatic stellate cells that produce excessive amounts of extracellular matrix proteins like collagens, a process that is well known as fibrosis (reviewed in (16)). The rigid extracellular matrix materials eventually encapsulates the liver lobules and progresses to cirrhosis and as a result, blood and bile flow in the liver become obstructed. Cirrhosis eventually leads to liver failure, with the only treatment option being liver transplantation. Chronic liver disease can develop unnoticed because the normal liver has a significant overcapacity. Symptoms may not become apparent until the liver function is reduced to below 30% of its original capacity. Common symptoms of liver disease are inflammation, jaundice (yellowing of the skin), darkened urine and light-colored stools, generalized itching, fatigue and abdominal pain in the upper right part of the stomach.

The liver plays an important role in sensing pathogens and hosts the largest population of resident tissue macrophages (Kupffer cells) (Figure 2A) as well as T lymphocytes, natural killer (NK) cells and natural killer T (NKT) cells (reviewed in (17)). The innate immune response plays an important role in hepatic damage after liver injury. For instance, absence of functional Toll like receptor 4 (TLR4), the endogenous sensor of the bacteria-derived endotoxin lipopolysaccharide (LPS), protect mice from alcohol-induced liver inflammation and damage (18). Furthermore, decreased recruitment of neutrophils decreases hepatic necrosis and improves survival in mice after acetaminophen-induced liver injury (19,20).

2.4 Modes of cell death during liver disease

Liver injury is associated with loss of function and/or viability of hepatocytes. Hepatocytes die, but different modes of cell death can be distinguished, e.g. necrosis and apoptosis (or programmed cell death). Necrosis is a passive, degenerative process. When cells are suddenly exposed to high levels of toxic compounds or different environmental conditions (e.g. extremely high or low temperatures or oxidative stress (21)), the integrity of the cell membrane collapses and the cell content is released into the environment. In vivo, this provokes an inflammatory reaction, often leading to even more tissue damage. In contrast, apoptosis is a tightly

regulated process. In short, apoptosis is initiated by extracellular or intracellular signals that cause an ATP-driven activation of proteases (caspases) that leads to cell shrinkage, chromatin condensation, mitochondrial dysfunction and cell fragmentation into apoptotic bodies. The apoptotic bodies remain membrane-surrounded and are removed by phagocytosis. In contrast to necrosis, apoptosis does not lead to massive release of cellular components into the circulation,

There are two major pathways of apoptosis depending on the origin of the apoptotic trigger, the extrinsic pathway and the intrinsic pathway (Figure 3) (reviewed in (22)). The extrinsic pathway requires activation and oligomerization of membrane-bound "death" receptors (e.g. Fas, TNF/TRAIL-receptors) by their corresponding agonists (Fas-Ligand, Tumor Necrosis Factor alpha, TRAIL). Extracellular activation of these death receptors results in the intracellular recruitment of adaptor proteins

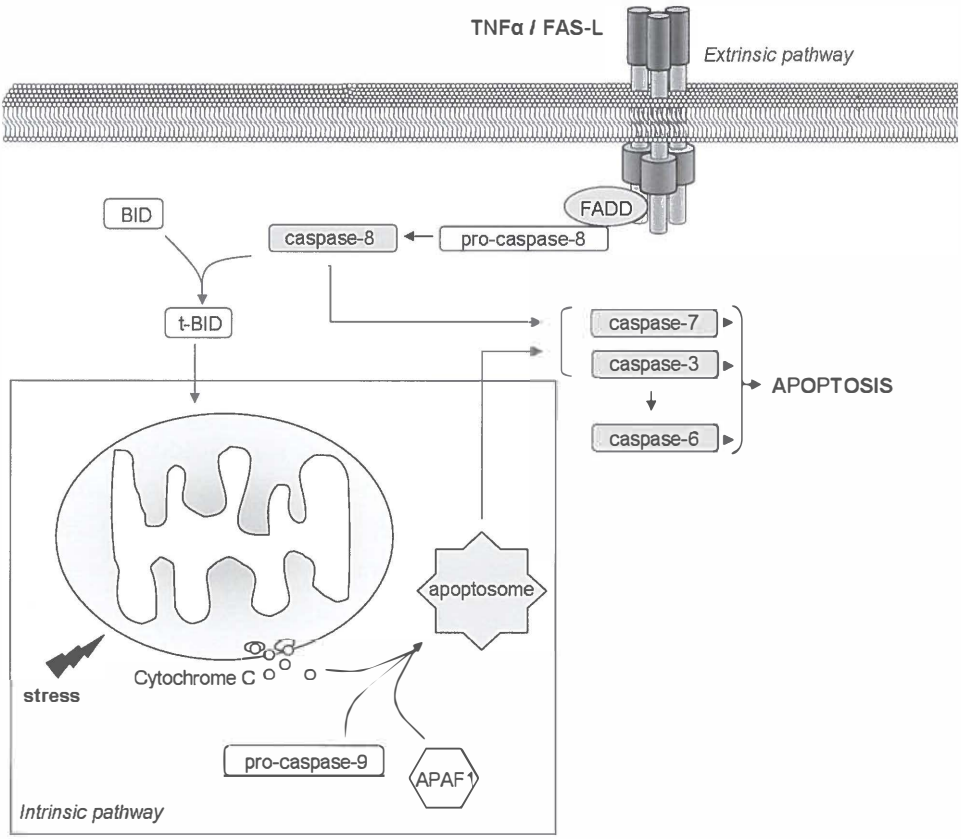

Figure 3: *Apoptosis pathways. Internal signals (e.g. oxidative stress) initiate the intrinsic pathway. The mitochondrial membrane destabilizes leading to leakage of cyochrome C. Cytochrome C in combination with procaspase-9 and APAF-1 form the apoptosome, thereby activating caspase-9. Caspase-9 activates caspase-3 and -7, leading to apoptosis.*
The extrinsic pathway is activated by binding of specific ligands to membrane-bound receptors. Activation leads to the intracellular recruitment of adaptor proteins, which activate caspase-8. Caspase-8 directly activates caspase-3 or -7 and leads to cytochrome C leakage from mitochondria.

and ultimately in the recruitment and cleavage of caspase-8. Cleaved and active caspase-8 is the initiator of the intracellular apoptotic cascade. Caspase-8 cleaves the protein BID (BH3-interacting domain death agonist) and activates the effector caspases, caspase-3 and -7. Truncated Bid translocates to mitochondria, where it induces pore formation in the mitochondrial membrane, resulting in leakage of cytochrome C into the cytoplasm. Cytochrome C than interacts with caspase-9 and the apoptotic protease-activating factor 1 (APAF1) to form the apoptosome. Subsequently, caspase-9 is activated and cleaves and activates caspases-3 and -7. The effector caspases 3 and 7 are proteases that attack and destroy numerous proteins, which will lead to the characteristic apoptotic cell death.

The intrinsic pathway is activated by intracellular signals. The main trigger for apoptosis is cellular stress (oxidative stress, protein misfolding, DNA damage). It typically activates one or more members of the BH3-only family (e.g. BID, BAD). When the amount of activated BH3-only members exceeds a certain threshold "organelle-damage"-specific caspases are activated (e.g. caspase-9 for mitochondria and caspase-12 for the endoplasmic reticulum) that lead to the activation of the downstream effector caspases (caspase-9, caspase-7). The execution phase of both intrinsic and extrinsic pathways of apoptosis are similar, as both pathways converge in activation of the effector caspases 3 and 7.

2.5 Liver regeneration

The liver possesses the remarkable ability to regenerate after injury. This process is very rapid, e.g. after surgical removal of 70% of the liver in mice, the organ grows back to it's original volume in only a few days (23).

Liver regeneration depends largely on proliferation of hepatocytes (reviewed in (24)). However, this is only true when there are no other confounding factors that affect their function and viability. In the healthy liver, only 1 in 20.000 to 40.000 hepatocytes is dividing. When liver tissue is lost, hepatocytes quickly respond and start to proliferate to restore the original liver mass. The capacity of hepatocytes to divide and restore liver mass is nicely illustrated by experiments in which a small amount of mature rat hepatocytes were transplanted into rats who were partially hepatectomized and previously treated with a chemical to block proliferation of resident hepatocytes. After 2 months, liver mass and function were back to normal and was composed predominantly of donor hepatocytes (25,26). In most liver diseases, however, hepatocyte proliferation is (partly) inhibited, e.g. due to viral infections, high levels of cytokines, bile salts, oxidative stress or chemical inhibition/drug intoxication. In these conditions, liver regeneration depends on the activation and differentiation of liver-specific stem cells and/or the recruitment of bone marrow stem cells. The

liver-specific stem cell compartment (also called hepatic progenitor cells) is located at the interface of the hepatocytes and the bile duct epithelial cells (cholangiocytes), also known as the canal of Hering (Figure 2 B). The hepatic progenitor cells are pluripotent and can differentiate into several liver-specific cell types, including the 2 main functional cell types, hepatocytes and cholangiocytes (27). Based on their morphological appearance, hepatic progenitor cells are also called oval cells.
Previous studies have shown that several ATP-binding cassette (ABC) transporters are induced in hepatic progenitor cells concomitant with the activation process. This was observed both in liver disease models in rats (28) and in severe liver disease in humans (29), suggesting an important function of these transporters in hepatic progenitor cells. In the following section, first a general overview of the ABC-transporter family is presented, followed by detailed information about MRP1.

3. ATP Binding Cassette transporters

The first ATP-binding cassette (ABC) transporter was identified in the beginning of the 1980's in research aimed at resolving the molecular mechanism that makes cancer cells resistant to chemotherapeutic drugs ((30-32), reviewed in (33)). The development of (multi)drug resistance is a common problem in the treatment of cancer. A protein was identified that is responsible for exporting the anti-cancer drug out of the cancer cell. This protein was called P-glycoprotein (P-gp), or the multidrug resistance protein 1 (MDR1). Soon after the first identification of MDR1, a structural homologue was found that shared many of its features and was named multidrug resistance-associated protein 1 (MRP1). MDR1 and MRP1 are now known as the founders of the ABC-transporter superfamily. Although MDRs and MRPs are particularly know for generating multidrug resistance in cancer cells, they are also expressed in normal human cells.
Many compounds are transported in, out and through cells, crossing cellular and organellar membranes. The family of ABC transporters play a key role in these processes, in particular in transporting potentially toxic compounds. The ABC transporter superfamily consists of 49 members divided into 7 subgroups (A-G) (reviewed in (34)). MDR1 is the prototypical ABC transporter and contains two transmembrane domains (TMD) connected by two intracellular nucleotide binding domains (NBD). The NBDs of the ABC transporters contain highly conserved amino acid sequences, the so called Walker A and B motifs and the ABC signature sequence (or C motif) that resides in between the Walker A and B motifs (Figure 4A). The A, B and C motifs are involved in binding and hydrolyzing ATP that drives the unidirectional substrate transport of ABC transporters. Proteins belonging to the ABC transporter superfamily are divided into subfamilies based on their structural

homology. Although most ABC transporters comply to the MDR1-type structure, there are variations to this. First of all, there are ABC-transporters that contain only one TMD and one NBD, e.g. ABCG5, G8 and ABCD1 to 4 (Figure 4B). These "half" transporters need to dimerize to form a fully functional transporter and is structurally similar to MDR1. Secondly, several of the MRP-type ABC-transporters of subfamily C contain a third transmembrane domain (TMD0) upstream of the core TMD1-NBD1-TMD2-NBD2, e.g. ABCC1/Multidrug resistance protein 1 (MRP1), ABCC2/ MRP2 and ABCC3/MRP3) (Figure 4C).

3.1 The ABCC subfamily and Multidrug Resistance-associated proteins

With 13 members, the ABCC subfamily is the largest subfamily of ABC transporters. It contains MRP1 to 10 (ABCC1-6, ABCC10-13), CFTR (cystic fibrosis transmembrane conductance regulator/ABCC7), SUR1 (sulfonylurea receptor/ABCC8) and SUR2A

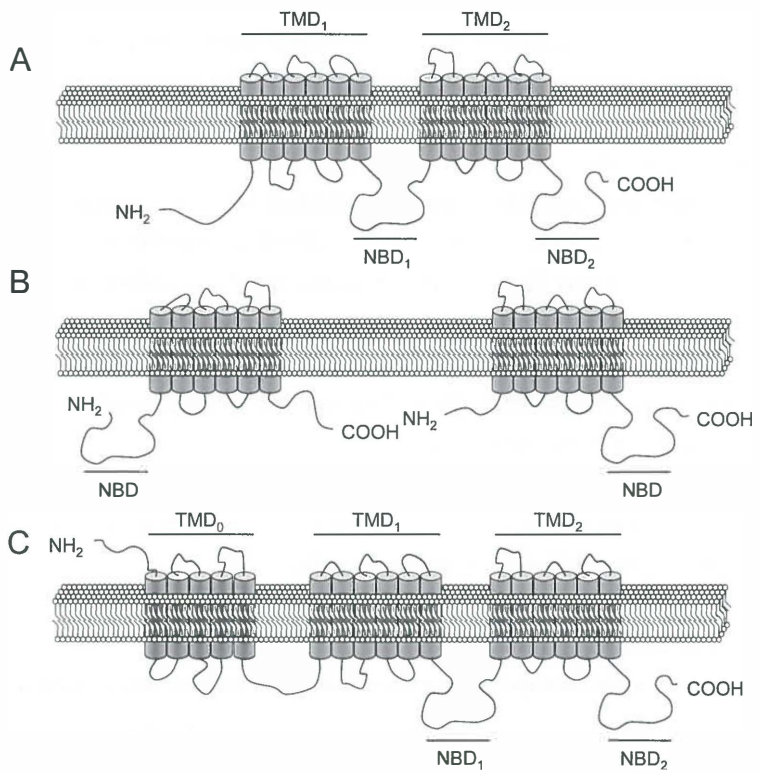

Figure 4. *Typical structures of ABC transporters. (A) standard structure of ABC transporters, containing 2 membrane spanning domains with an intracellular amino and carboxylic terminal domain. Two intracellular nucleotide binding domains are present. (B) Structure of ABC half-transporters. These transporters consist of one TMD, one NBD and intracellular amino and carboxylic terminal domains. (C) Structure of "long" ABC transporters from the ABCC subfamily. These transporters contain an additional TMD at the amino terminal side of the protein. The amino terminal domain is located extracellularly.*

and B (ABCC9) (reviewed in (35)). All MRPs, have a distinctly different structural element compared to other ABC transporters. All MRPs show different spacing between the Walker A and the ABC signature motif in the NH2-proximal NBD (NBD1) compared to the COOH-proximal NBD (NBD2). Moreover, members of the ABCC subfamily contain a highly conserved single amino acid substitution (glutamate to aspartate) in the NBD1, just following the Walker B motif, that differentiates them from other ABC transporters (36,37). This substitution strongly affects the catalytic properties of NBD1. A glutamate residue at that position, as found in other ABC transporters, enhances the ATPase activity of this NBD. However, the substrate transport activity of MRP1 is severely impaired when the glutamate is introduced by site directed mutagenesis (36). In ABCC transporters, ATP hydrolysis takes place mainly at NBD2, whereas NBD1 is responsible for ATP binding. In contrast, NBD1 and NBD2 in other ABC transporters are involved in both ATP binding and hydrolysis. CFTR, SUR1 and SUR2 are ion channels (chloride and potassium, respectively), whereas the MRP1-MRP8 genes all encode transporters responsible for transport of bulky compounds. The functions of MRP9 and MRP10 are still a matter of debate. In fact, it is unclear whether the human ABCC13 gene (encoding MRP10) produces a functional protein (38).

The ABCC subfamily can be further subdivided into two groups, the long and short ABCC proteins. CFTR, MRP4, MRP5, MRP8 and MRP10 have a "standard" structure consisting of two TMD's and two NBDs. MRP1-3, MRP6, MRP7, SUR1 and SUR2 contain the extra TMD0 at the NH2 terminus (reviewed in (35)). MRP9 is an exception, as the gene encodes two proteins, the first consists of 8 transmembrane domains and lacks the second NBD. The second smaller protein is expected to encode for only the second NBD (39).

MRP1 and MRP3 are the closest relatives based on the amino acid sequence and share 58% amino acid identity. MRP1 shares 45% and 43% amino acid identity with MRP2 and MRP6, respectively (reviewed in (35)).

3.2 ABC transporters in the intestinal epithelium.

Many ABC transporters are expressed in the enterocytes of the intestinal epithelium. On the one hand they provide protection against potentially toxic compounds in the gut lumen. On the other hand, they are involved in the efficient absorption of nutrients and transport to the circulation. Particularly MDR1 and MRP3 are expressed at high levels in the intestinal epithelium. MDR1 is located at the apical membrane of the enterocyte and transports neutral and positively charged amphiphatic compounds towards the gut lumen. It is an important factor in the intestinal epithelium that protects the organism from these toxic compounds in the gut. Like hepatocytes,

enterocytes are also involved in the detoxification of toxins. MRP3 is (one of) the most dominant MRP-type transporter in enterocytes and is involved in the export of conjugated metabolites to the blood, so they can be excreted via the kidneys (40-42). MRP1, MRP4, MRP5 and MRP6 are also present in enterocytes in the healthy intestine and may aid in the secretion of such compounds (reviewed in (35)). The ABCG5/ABCG8 heterodimer resides in the apical membrane of enterocytes and the hepatocytes and exports plant sterols to the gut lumen or the bile thereby preventing their accumulation in blood (43,44).

3.3 ABC transporters in hepatocytes

ABC transporters that play a crucial role in the physiological function of the liver are the bile salt export pump (BSEP, ABCB11), the multidrug resistance protein 3 (MDR3/ABCB4; Mdr2 in rodents) and MRP2 (ABCC2). These three proteins reside in the canalicular membrane of hepatocytes and transport substrates to the bile (reviewed in (34)). BSEP and MDR3 act together in the formation of bile. BSEP is the main transporter for bile salts, while MDR3 transports phospholipids. Bile salts and phospholipids form mixed micelles in bile and are the carriers for fatty compounds that need to be excreted or absorbed in the intestine. MRP2 is the transporter for bilirubin (45), the breakdown product of blood hemoglobin. In addition, MRP2 excretes divalent sulphated or glucuronidated bile acids that may accumulate during liver disease (46). MRP1, MRP3 and MRP4 are also expressed in the liver, however, at considerably lower levels compared to BSEP, MDR3 and MRP2. They reside in the basolateral membrane. Expression of MRP3 and MRP4 is induced in hepatocytes during cholestatic liver disease and transport bile acids into the blood. This is considered a rescue mechanism that comes into play to protect hepatocytes from bile salt-induced cell damage. MRP1 levels are low in the healthy liver, but are induced during liver disease and is particularly present in the hepatic progenitor cell compartment (29).

3.4 MRP substrates

MRPs transport a wide variety of substrates, but the individual substrate specificity varies greatly. MRP1 and MRP2 share a considerable substrate overlap, transporting predominantly organic anions such as glucuronide- or sulphate-conjugates (phase 2 metabolites). Examples of well known MRP1/MRP2 substrates are leukotriene C4, steroids, bile salt-metabolites and estrogen conjugates. In contrast, MRP3 transports unconjugated bile salts as well as a wide variety of glucuronide conjugates. Substrates for MRP4 and MRP5 are nucleoside and nucleotide analogs and cyclic nucleotides. Furthermore, MRP4 and MRP6 have recently been shown to transport

leukotriene C4 with equal specificity as MRP1 (47,48).

3.5 MRP cellular location

MRPs are ubiquitously expressed in the healthy body. However, a distinct difference is observed in the abundance of specific MRPs in specific tissues. MRP1 expression is high in lung, testis, kidney, placenta, skeletal and cardiac muscle (36,49,50) and low in the liver and intestine. MRP2 is expressed in liver, kidney, small intestine, colon, gall bladder, placenta, and lung while MRP3 is expressed mainly in the adrenal gland, pancreas, gut, gall bladder, and placenta. Tissue distribution of all MRP-type transporters are shown in Table 1.

MRP-type transporters are all efflux transporters. However, the direction of transport towards specific body fluids varies depending on their cellular location. Generally, MRPs are located on the cell membrane transporting compounds from the cytosol into the cell environment. However, most cells found in the body are embedded in

Table 1: *Tissue distribution, subcellular location and endogenous substrates for MRP type ABC transporters (reviewed in (34,51))*

MRP	Organ	Membrane location	Substrates
MRP1/ABCC1	ubiquitous; high in lung, brain, heart, testis	Basolateral	LTC_4, glutathione-, sulphate- and glucuronide conjugates, glutathione
MRP2/ABCC2	liver, kidney	Apical	LTC_4, estradiol glucuronide, bilirubin glucuronide
MRP3/ABCC3	Liver	Basolateral	LTC_4, estradiol glucuronide, glutathione
MRP4/ABCC4	Brain, brain capillary endothelium	Basolateral	conjugated steroids, cAMP, cGMP, bile salts, prostaglandins
MRP5/ABCC5		Basolateral	conjugated steroids, cAMP, cGMP, bile salts
MRP6/ABCC6	liver , kidney	Basolateral	Glutathione conjugates, LTC_4
MRP7/ABCC10	ubiquitous; high in pancreas	Unknown	estradiol glucuronide
MRP8/ABCC11	foetal tissue, central and peripheral nervous system	apical (predicted)	cyclic nucleotides, steroid sulphates
MRP9/ABCC12	Ubiquitous; mainly testis, brain, ovary, prostate	endoplasmic reticulum	Unknown
MRP10/ABCC13	Pseudogene; encodes a non-functional ABC transporter		

organs facing two different environments. These cells are polarized and developed two different subdomains in the plasmamembrane. The plasmamembrane facing the blood is called the basolateral membrane, membranes facing away from the blood are called apical (or canalicular in the liver) membranes. In these polarized cells, MRPs are differentially distributed across the cellular membrane. MRP1, MRP3 and MRP4 are located at the basolateral membrane, whereas MRP2 is exclusively located at the apical membrane.

4 MRP1 function in (patho)physiological conditions

4.1 Discovery of MRP1

MRP1 was first described in 1992 (36). The ABCC1 gene that encodes MRP1 was cloned and sequenced from a doxorubicin resistant human lung cancer cell line. The substrate specificity of MRP1 has been extensively studied. MRP1 transports a wide variety of (metabolites of) chemotherapeutic drugs, thereby limiting the efficacy of drug-based cancer therapy (reviewed in (52-55)). These drugs include Vinka alkaloids, anthracyclines and platinum-based drugs.

4.2 Endogenous substrates and their role in inflammation of the gut and liver.

MRP1 has a high specificity for glucuronide-, sulphate- and glutathione-conjugates that are generated in the cellular detoxifying machinery (also known as phase-2 metabolites) (56-58). This function is in fact much like its role in multidrug resistance in cancer cells, but now protecting cells in normal tissue. MRP1 transports reduced and oxidized glutathione ((59), reviewed in (60)). Glutathione is involved in the cellular protection against oxidative stress and export of oxidized glutathione provides cellular against cell death. MRP1 also transports the pro-inflammatory cytokine leukotriene C_4 (58,61)(Figure 5), suggesting a possible role of MRP1 in inflammatory reactions. MRP1 is present in several inflammatory cell types in peripheral blood (62-64). Mrp1 expression is induced in activated Th-lymphocytes (62,65). In addition, blocking Mrp1 function inhibits Th-lymphocyte activation and significantly alters the cytokine release profile (63). This observation is supported by the fact that Mrp1-deficient mice show a disturbed response to inflammatory stimuli (66). Moreover, Mrp1-deficient mice are more susceptible to develop colitis compared to wild type animals (67). Thus, MRP1 may be an important factor in the intestinal epithelium that protects against the development of Inflammatory Bowel Disease.

Specific induction of MRP1 was also detected in human inflammatory liver diseases like primary biliary cirrhosis and chronic hepatitis C infection. While MRP1 expression in the healthy liver is low, MRP1 expression is clearly induced in hepatocytes and

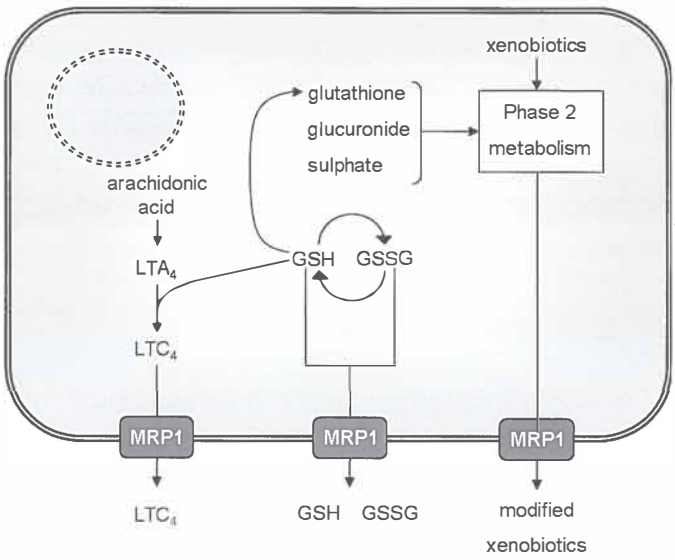

Figure 5. *Main physiological substrates of MRP1. MRP1 has a high affinity for the pro-inflammatory cytokine leukotriene C4, glutathione and phase 2 metabolites of xenobiotics and endogenous toxins.*

ductules in the inflamed liver. Upregulation of MRP1 was particularly evident in the progenitor cell compartment (29). This observation points towards a possible role for MRP1 in hepatic progenitor cell activation and/or function.

4.3 MRP1 transcriptional regulation

Chemotherapeutic drugs such as Vinca-alkaloids (68), anthracyclines, etoposide (66,69), platinum based drugs (68) and epipodophyllotoxines may lead to induced expression of MRP1 in cancer cells and cause multidrug resistance. Detailed knowledge about the transcriptional regulation of MRP1 is required to understand the underlying mechanisms that could be used to limit MRP1 expression and thereby prevent the problem of drug resistance. Unfortunately, relatively little is known of transcriptional regulation of MRP1. The human MRP1 promoter has been cloned and putative binding sites have been identified for Activator Protein 1 (AP-1 , a complex of the proteins c-jun and c-fos) and Sp1 transcription factor (70). In addition, responsive elements have been identified, including a cyclic AMP response element (CRE), a glucocorticoid response element (GRE) and multiple estrogen response elements (ERE) (71).

Although several binding sites were identified there is no direct evidence that the related transcription factors actually modulate MRP1 transcription.

In addition, oxidative stress was shown to increase MRP1 expression in rat hepatoma cells (72), but there is no information yet about the factors or regulatory mechanisms involved.

Besides chemotherapeutic drugs, numerous endogenous compounds have been

identified that are able to regulate MRP1 expression. The human tumor suppressor protein p53 (70,73), Sp1 transcription factor (73,74) as well as the growth factor epidermal growth factor (EGF) (75) are able to modulate the expression of MRP1 in human cancer cells. Sp1 is a strong activator of MRP1 expression. Sp1 is able to induce MRP1 expression up to 200-fold (74,76). In the presence of p53, transcription activators are not able to bind to Sp1 sites of the MRP1 promoter. It is hypothesized that p53 directly binds Sp1, thereby suppressing its activity (77). Treatment of MCF-7 breast cancer cells with EGF shows an increased expression of the MRP1, 3, 5 and 7 genes and increased MRP1 promoter activity (75). Furthermore, treatment of mice with peroxisome proliferator-activated receptor (Ppar)-alpha agonists showed reduced intestinal expression of Mrp1, indicating a possible role for Ppar-alpha in Mrp1 regulation (78). PPARs are involved in the regulation of fatty acid metabolism. PPAR-alpha is the most dominant PPAR in the liver, regulating transcription of genes involved in fatty acid-induced catabolism. However, no direct evidence is available for the involvement of these transcription factors in the regulation of MRP1 transcription. The mechanism by which MRP1 is regulated in physiological conditions is therefore still obscure.

4.4 MRP1 function during (smoke-induced) inflammation

Recently, extensive evidence has been obtained for a role of MRP1 in cytoprotection in physiologically relevant conditions. Van der Deen et al., demonstrated that MRP1 protects against negative effects of smoking. They show that MRP1 inhibition aggravates cigarette smoke-induced cell death in human lung epithelial cells (79). In addition, Mrp1/Mdr1a/Mdr1b triple knockout mice showed a reduced inflammatory response to cigarette smoke (79,80).

Taken together, these observations indicate that MRP1 may have an important function during inflammation. MRP1 absence leads to reduced export of leukotriene C4, which is involved in maintaining the inflammatory response as part of the so called slow reaction substance of anaphylaxis. By blocking MRP1 mediated export, LTC4 is retained in the cells and leads to the formation and secretion of LTB4, which is a chemotactic factor for neutrophils. Inhibition of MRP1 may therefore change the inflammatory cascade from a T cell-mediated reaction to a neutrophil-mediated reaction.

5. Scope of the thesis

The aim of this thesis is to determine the function and regulation of the multidrug resistance-associated protein 1 (MRP1) in diseases of the intestine and the liver. In chapter 2, we compared the expression of mucosal MRP1 in uninflamed and inflamed

regions of the terminal ileum and colon of patients with inflammatory bowel disease (IBD). As MRP1 is significantly induced by inflammation, we analyzed its protective role in intestinal epithelial cells exposed to various inflammatory signals. In chapter 3, we extended our analysis to the function of MRP1 in T-lymphocytes in patients with IBD. T-lymphocytes are overactivated in IBD and often resistant to drug-induced cell death. Therefore, we compared the expression of MRP1 in T-lymphocytes from healthy volunteers and IBD patients and analyzed its role in protecting these cells against apoptotic cell death. In chapter 4, we changed our focus to the liver and determined the function of Mrp1 in stem cell-driven liver regeneration in mice. Liver regeneration was induced by removing 70 % liver mass from wild type and Mrp1-knock out mice that were simultaneously treated with 2-acetylaminofluorene to block hepatocyte proliferation. Liver regeneration than depends on proliferation and differentiation of stem cells. Finally, in chapter 5, we studied the putative involvement of PPAR-alpha in the transcriptional regulation of human MRP1 colon and hepatic cell lines. PPAR-alpha is present in the liver and intestine and its activity can be manipulated by various pharmaceutical agonists and antagonists. It is therefore an interesting target to regulate MRP1 expression in liver and intestinal diseases.

Taken together, this thesis aims to increase our understanding of the role and regulation of MRP1 in intestinal and hepatic diseases to help develop and/or improve the treatment of these patients.

6. References

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CHAPTER 2

Upregulation and cytoprotective role of epithelial Multidrug Resistance-associated Protein 1 in inflammatory bowel disease

J. Biol. Chem. 283 (2008), 35630-35637

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ABSTRACT

The multidrug resistance-associated protein 1 (MRP1) is well known for its role in providing multidrug resistance to cancer cells. In addition, MRP1 has been associated with both pro- and anti-inflammatory functions in non-malignant cells. The pro-inflammatory function is evident from the fact that MRP1 is a high-affinity transporter for cysteinyl-leukotriene C_4 (LTC_4), a lipid mediator of inflammation. It remains unexplained, however, why the absence of Mrp1 leads to increased intestinal epithelial damage in mice treated with dextrane-sodium sulphate, a model for inflammatory bowel disease (IBD). We found that MRP1 expression is induced in the inflamed intestine of IBD patients, e.g. Crohn's disease and ulcerative colitis. Increased MRP1 expression was detected at the basolateral membrane of intestinal epithelial cells. To study a putative role for MRP1 in protecting epithelial cells against inflammatory cues, we manipulated MRP1 levels in human epithelial DLD-1 cells and exposed these cells to cytokines and anti-Fas. Inhibition of MRP1 (by MK571 or RNA interference) resulted in increased cytokine- and anti-Fas-induced apoptosis of DLD-1 cells. Opposite effects, e.g. protection of DLD-1 cells against cytokine- and anti-Fas-induced apoptosis, were observed after recombinant MRP1 overexpression. Inhibition of LTC_4 synthesis reduced anti-Fas-induced apoptosis when MRP1 function was blocked, suggesting that LTC_4 is the pro-apoptotic compound exported by epithelial MRP1 during inflammation. These data show that MRP1 protects intestinal epithelial cells against inflammation-induced apoptotic cell death and provides a functional role for MRP1 in the inflamed intestinal epithelium of IBD patients.

INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract and manifests as ulcerative colitis (UC) or Crohn's disease (CD) (1). The pathophysiology of IBD is characterized by a highly activated state of the mucosal immune system and excessive mucosal damage (2). In recent years, important progress has been made in identifying and characterizing susceptibility genes for IBD (3). The (putative) functions of the proteins they encode corroborate the notion that the primary cause for the development of IBD originates from a dysregulated immune response to commensal intestinal bacteria, defects in mucosal barrier function and/or bacterial clearance.

Irrespective of the genetic cause, severe and continuous inflammation causes damage to the intestinal epithelium that may strongly affect its absorptive and secretory functions as well as its protective role against toxic compounds. Cytoprotection is provided by ATP-binding cassette (ABC) transporters that are specialized in exporting toxic compounds of foreign or endogenous origin (4). P-gp/ MDR1 (ABCB1), well-known for its adverse role in protecting cancer cells against anti-cancer drugs (5), is also expressed in the healthy intestinal epithelium (6). Absence of *Mdr1a* in transgenic mice results in the spontaneous development of colitis (7). In line with this observation, specific single nucleotide polymorphisms (SNPs) in the *ABCB1* gene have been reported to be associated with CD (8-10). Moreover, we recently showed that MDR1 expression is strongly reduced in the inflamed intestine of IBD patients, which may further aggravate the disease (11).

The multidrug resistance-associated protein 1 (MRP1, encoded by the *ABCC1* gene) also plays a role in inflammatory responses. It transports glutathione and substrates that are conjugated to reduced glutathione, glucuronide or sulphate as part of the detoxification machinery of (cancer) cells (12;13). The glutathione conjugate leukotriene C_4 (LTC_4) has been identified as an endogenous, high affinity substrate for MRP1(14;15). Leukotrienes are important lipid mediators of inflammatory responses and have been implicated in the pathophysiology of both acute and chronic inflammatory diseases, including IBD (14;16;17). The role of Mrp1 in the inflammatory signalling pathway is evident from studies with $Mrp1^{-/-}$ knockout mice that show a strongly reduced response to arachidonic acid-induced inflammatory stimuli as measured by decreased ear oedema and vascular permeability (18). Relevant for IBD, however, it was shown that intestinal damage was significantly aggravated in $Mrp1^{-/-}$ knock-out mice exposed to dextrane sulfate sodium (DSS)-induced colitis (19). These animal studies suggest that Mrp1 serves a dual role

during inflammation, both sending out inflammatory signals as well as protecting the intestinal epithelium. The mechanism of the latter and the relevance for IBD patients is unknown to date.

In this study, we show that MRP1 expression is increased in the inflamed epithelium of patients with IBD, in particular in the intestinal crypts. We demonstrate that MRP1 protects intestinal epithelial cells against cytokine-induced cell death by exporting pro-apoptotic compounds from the cysteinyl leukotriene biosynthesis pathway.

MATERIALS AND METHODS

Patient characteristics

Intestinal mucosal biopsy specimens were obtained during endoscopy following informed consent from patients with IBD. Diagnosis of IBD was established by endoscopic and histopathological examination. The Ethics Committee of the University Hospital Groningen approved the protocol (METc 2002/177c). Intestinal biopsies were obtained from macroscopically inflamed and non-inflamed mucosa from 35 patients with IBD using a standard biopsy forceps. Intestinal specimens were immediately snap-frozen in liquid nitrogen for mRNA and protein analysis or liquid nitrogen-cooled isopentane for immunohistochemical staining, and stored at –80 °C until further processing.

Immunohistochemistry

Immunohistochemistry was performed with the mouse monoclonal antibody QCRL-3 against MRP1 on frozen sections according to the protocol described (dilution 1:10, Santa Cruz Biotechnology, Heidelberg, Germany) (20). The sections were counterstained with hematoxylin. Negative controls consisted of omission of the primary antibody and were consistently negative.

Cell culture

The human colon carcinoma cell line DLD-1 was cultured as previously described (21). DLD-1 cells were treated with anti-Fas (Immunotech, Marseille, France) or human tumor necrosis factor-α (TNF-α, R&D Systems Europe, Abingdon, UK). The LTD_4 receptor antagonist MK571 (3-([[3-(2-[7-chloro-2-quinolinyl]ethenyl) phenyl}-((3-(dimethylamino-3-oxopropyl)-thio)-methyl]thio) propanoic acid; Alexis Biochemicals, Lausen, Switzerland) was used to inhibit MRP function. The 5-lipoxygenase inhibitor AA861 (2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-p-benzoquinone; Sigma, Zwijndrecht, The Netherlands) was used to inhibit leukotriene synthesis from

arachidonic acid.

siRNA-mediated reduction of MRP1 expression

DLD-1 cells were seeded in 6 well plates at a density of 400.000 cells per well in RPMI 1640 with glutamax supplemented with 1 % fetal bovine serum (FBS). After 4 hours, medium was replaced by serum free medium. DLD1 cells were transiently transfected with siRNA-MRP1 duplexes using oligofectamine (Invitrogen, Breda, The Netherlands) according to the manufacturers instructions. SiRNA-MRP1 primers were obtained from Invitrogen (sense 5'-GGA GUG GAA CCC CUC UCU GdTdT-3' and antisense 5'-CAG AGA GGG GUU CCA CUC CdTdT-3'; kindly provided by Dr. D.J. de Groot, University Medical Center Groningen, Groningen, The Netherlands). Control cells were treated with only oligofectamine. Four hours after transfection, medium was supplemented with RPMI-1640 with 30 % FBS to adjust the serum concentration to 10 %. Subsequently, the cells were cultured in an incubator at 37 °C and 5 % CO_2 for 48 hours. MRP1 RNA and protein expression was then analyzed by real time PCR and Western blot analysis.

MRP1 overexpressing cell line

DLD-1 cells were seeded in 6-well plates at 300.000 cells per well. The cells were transfected with plasmid pcDNA3.1(-)-MRP1-GFP (22). Transfection was performed using the BIO-RAD Transfectin™ Lipid reagent protocol (Bio-Rad laboratories B.V., Veenendaal, The Netherlands). Transfected cells were grown to 70 % confluence before adding 0.625 mg/ml geneticine to the medium. After three days colonies were selected and expanded in medium containing 0.625 mg/ml geneticine. Two clones were further purified by fluorescence activated cell sorting (FACS) based on the GFP signal to obtain homogenous MRP1-expressing cell lines.

RNA isolation and quantitative PCR

Total RNA was isolated as described (23). RNA integrity was confirmed by agarose gel electrophoresis and RNA concentration was measured using a Ribogreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). Complementary DNA (cDNA) synthesis was performed on 2.5 µg of total RNA using random primers in a final volume of 50 µl (Reverse Transcription System, Promega, Madison, WI, USA). For real-time PCR, 4 µl of 20-fold diluted cDNA was used for every PCR reaction in a final volume of 20 µl, containing 900 nmol/L sense and antisense primers, 200 nmol/L fluorogenic probe, 5 mmol/l $MgCl_2$, 0.2 mmol/l deoxynucleoside triphosphate mix, 2 µl real-time PCR buffer (10x), and 0.5 U Hot Goldstar DNA Polymerase (Eurogentec,

Seraing, Belium). Details about primers and probes are described in Table S1. Real-time PCR was performed with an ABI Prism 7700 Sequence Detector version 1.6 software (Perkin Elmer Life Sciences, Foster City, CA, USA). The expression of genes was normalized to the endogenous control (18S).

Western blot

Total cell lysates of DLD-1 cells were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting. Protein concentrations were determined using the Bio-Rad *DC* Protein Assay

Table 1. **Sequences of primers and probes used for real-time detection PCR analysis**

18S	sense	5'-CGG CTA CCA CAT CCA AGG A-3'
	antisense	5'-CCA ATT ACA GGG CCT CGA AA-3'
	probe	5' FAM-CGC GCA AAT TAC CCA CTC CCG A-TAMRA 3'
iNOS	sense	5'-GGC TCA AAT CTC GGC AGA ATC-3'
	antisense	5'-GGC CAT CCT CAC AGG AGA GTT-3'
	probe	5' FAM-TCC GAC ATC CAG CCG TGC CAC-TAMRA 3'
MDR1	sense	5'-GGC AAA GAA ATA AAG CGA CTG AA-3'
	antisense	5'-GGC TGT TGT CTC CAT AGG CAA T-3'
	probe	5' FAM-CGT GTC CCA GGA GCC CAT CCT GT-TAMRA 3'
MRP1	sense	5'-CTT CTG GAG GAA TTG GTT GTA TAG AAG-3'
	antisense	5'-GGT AGA CCC AGA CAA GGA TGT TAG A-3'
	probe	5' FAM-TCT TTG AGA TGC TTC TGG CTC CCA TCA C-TAMRA 3'
MRP2	sense	5'-TGC AGC CTC CAT AAC CAT GAG-3'
	antisense	5'-CTT CGT CTT CCT TCA GGC TAT TCA-3'
	probe	5' FAM-CAG CTT TCG TCG AAC ACT TAG CCG CA-TAMRA 3'
MRP3	sense	5'-GCC ATC GAC CTG GAG ACT GA-3'
	antisense	5'-GAC CCT GGT GTA GTC CAT GAT AGT G-3'
	probe	5' FAM-CAT CCG CAC CCA GTT TGA TAC CTG CAC-TAMRA 3'
MRP4	sense	5'-AAG TGA ACA ACC TCC AGT TCC AG-3'
	antisense	5'-GGC TCT CCA GAG CAC CAT CT-3'
	probe	5' FAM-CAA ACC GAA GAC TCT GAG AAG GTA CGA TTC CT-TAMRA 3'
MRP5	sense	5'-TGA AAG CCA TTC CAG TTG-3'
	antisense	5'-CGG AAA AGC TCG TCA TGC A-3'
	probe	5' FAM-CTC GCA GCG TGC CCT TGA CAA AG-TAMRA 3'
MRP6	sense	5'-AGA CAC GGT TGA CGT GGA CAT-3'
	antisense	5'-GCT GAC CTC CAG GAG TCC AA-3'
	probe	5' FAM-CCA GAC AAA CTC CGG TCC CTG CTG AT-TAMRA 3'

system (Bio-Rad GmbH) using bovine serum albumin as standard. Rabbit polyclonal antibody against human PARP (dilution 1:1000, Cell Signaling Technology, Beverly, Massachusetts, USA), mouse monoclonal antibody GAPDH (dilution 1:5000, Calbiochem, Darmstadt, Germany) and rat monoclonal antibody MRPr1 were used (dilution 1:500) (24). Horse radish peroxidase-labeled swine anti-rabbit and rabbit anti-mouse IgG were used as secondary antibodies (dilutions 1:2500; DAKO, Heverlee, Belgium).

The blots were exposed in a ChemiDoc XRS system (Bio-Rad, Hercules, CA). The intensity of the protein bands were quantified using Quantity One software.

Caspase-3 assay

Caspase-3 enzyme activity was determined according to the manufacturer's instructions using a caspase-3 activity kit with fluorimetric detection (Promega, Leiden, The Netherlands).

Data analysis

Statistical analyses were performed using SPSS version 12.0 for Windows (SPSS Inc., Chicago, USA). Data obtained from the different groups are expressed as mean values ± standard deviation (SD). Different groups were compared using Mann-Whitney U-tests and Kruskal-Wallis tests. A p-value of <0.05 was considered as statistically significant.

RESULTS

Expression of MRP1 in inflamed and uninflamed intestinal tissue.

To study the effect of inflammation on ABC-transporter expression in the intestinal epithelium, we analyzed bordering mucosal biopsies from uninflamed and inflamed tissue from patients with IBD. In an initial screening, expression of MRP1-6 and MDR1 was analyzed in biopsies from 5 IBD patients (3 x UC and 2 x CD). MRP1 mRNA levels were significantly increased in inflamed intestinal mucosa, while MDR1 expression was down-regulated (Fig. 1A), as described earlier (11). The mRNA levels of MRP2-6 were similar in uninflamed and inflamed intestinal tissue of IBD patients (Fig. 1A). Subsequently, biopsies from 15 UC patients and 20 CD patients were analyzed. Inducible nitric oxide synthase (iNOS) expression was determined as a measure of inflammation in the biopsies (Fig. 1B). Also in this larger cohort of IBD patients, MRP1 expression was significantly increased in inflamed intestinal mucosa in both UC and CD (Fig. 1C) and MDR1 expression was decreased in both IBD subgroups (Fig. 1D). Immunohistochemistry for MRP1 on colonic biopsy material

with high grade inflammation showed a clear basolateral staining of intestinal epithelial cells as well as MRP1-positive mononuclear cells. Notably, MRP1 staining intensity was homogeneous in the epithelial cells lining the whole crypt, while the surface epithelium showed minimal MRP1-specific staining (Fig. 1E). In parallel

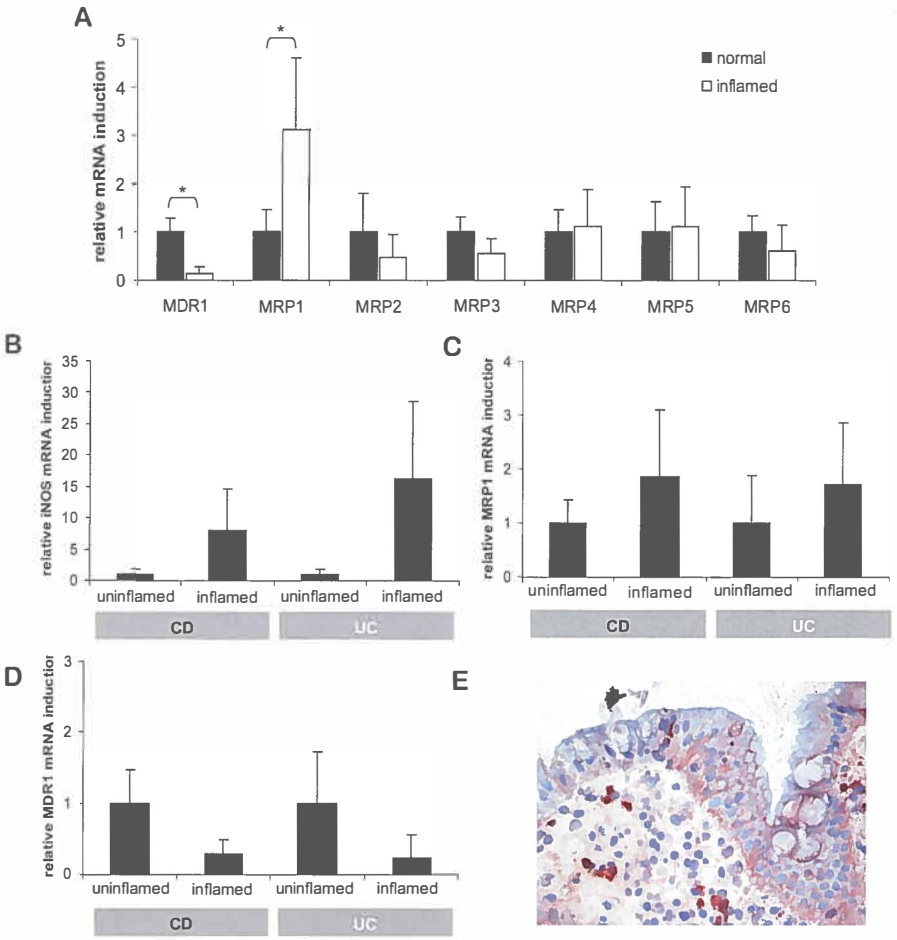

Figure 1. ***Increased MRP1 mRNA and protein expression in inflamed intestinal tissue of CD and UC patients.*** *(A) mRNA levels of MDR1 and MRP1-6 in uninflamed (normal) and inflamed tissue of 5 IBD patients (CD n=2; UC n=3) were determined by real time RT-PCR. Only for MRP1, a significant increase in inflamed intestinal tissue was detected. (B-D) mRNA levels of iNOS (B), MRP1 (C) and MDR1 (D) in uninflamed and inflamed tissue of 20 CD and 15 UC patients. MRP1 levels are increased in inflamed intestinal tissue of both patient groups. mRNA levels from uninflamed tissue was set to 1 and relative mRNA expression levels were normalized to 18S. Data are expressed as means ± SD. * $p<0.05$ compared with non-inflamed samples. (E) Immunohistochemical localisation of MRP1 expression in inflamed colonic tissue of a patient with ulcerative colitis. MRP1-dependent staining is indicated by arrowheads. The original magnification is 200x.*

experiments, the uninflamed tissue from these patients remained unstained for MRP1 (data not shown).

The effect of MK571-mediated inhibition of MRPs on anti-Fas- and TNF-α-induced apoptosis.

Increased MRP1 levels may aid to cell protection during gastrointestinal inflammatory diseases. Cell damage/death may be induced through receptor-mediated apoptosis in response to FasL or TNF-α, as previously shown in the intestinal mucosa of IBD patients (25-27). To study this putative function, we performed *in vitro* experiments using the human colon epithelial cell line DLD-1. Both human colon biopsies and DLD-1 cells show significant expression of MDR1 and various MRPs, including MRP1 (Fig 2). This makes DLD-1 cells a useful model for MRP1 inhibition and overexpression studies. First, we tested whether chemical inhibition of MRP-function by MK571 affected DLD-1 cell survival during exposure to anti-Fas or TNF-α. As reported by others (28-31), anti-Fas and TNF-α induce apoptosis in DLD-1 cells in a time-dependent manner with caspase-3 activities peaking at 16 and 12 hours for TNF-α and anti-Fas, respectively (Fig. 3A,D). Concurrently, significant amounts of activated (cleaved) PARP were detected after 16 hours TNF-α (Fig. 3B) or 8 hours anti-Fas (Fig. 3E) treatment. Adding MK571 strongly increased caspase-3 activities in both TNF-α- and anti-Fas-treated cells (Fig. 3A, D), which was accompanied by a further increase in the amount of cleaved PARP (Fig. 3B, E). The increase of TNF-α- or anti-Fas-induced apoptosis was dependent on the concentration of MK571 causing a significant increase of TNF-α- and anti-Fas-induced apoptosis at

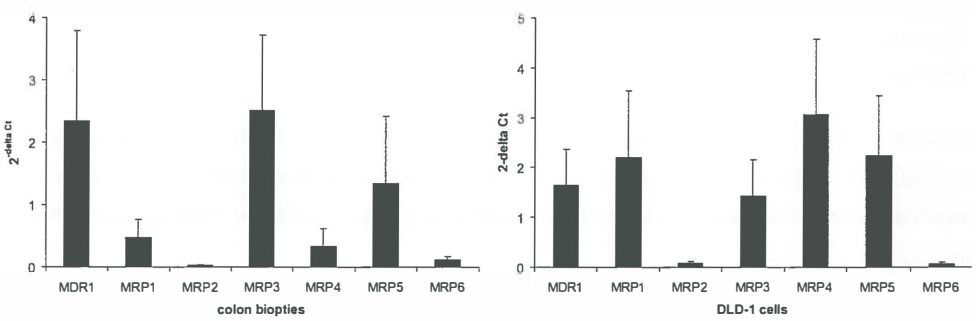

Figure 2. ***MDR1 and MRP1-6 mRNA expression in human colon biopsies and DLD-1 cells.*** *Relative mRNA levels of indicated genes were determined by real time RT-PCR and are corrected for 18S expression. Data are expressed as means ± SD.*

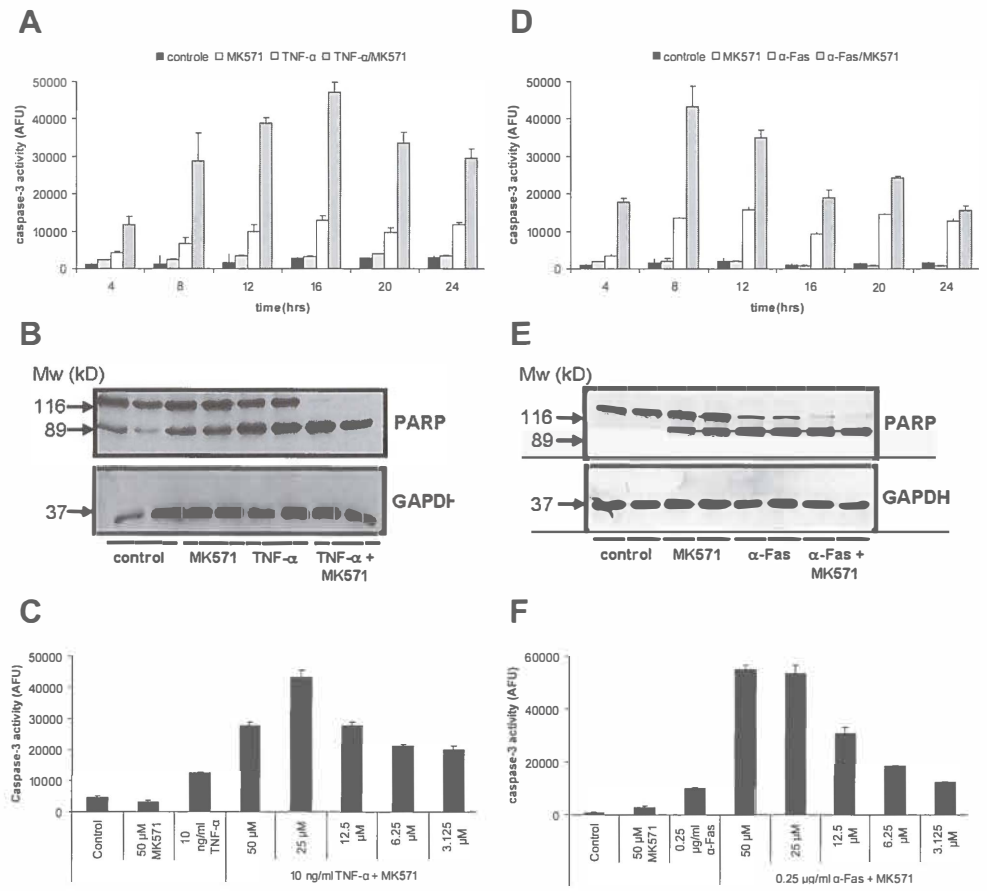

Figure 3. ***Effect of MK-571 on anti-Fas- and TNF-α-induced apoptosis of DLD-1 cells.*** *(A, D) Caspase-3 activity in DLD-1 cells treated for 4 to 24 hours with 10 ng/ml TNF-α (A) or 0.25 µg/ml anti-Fas (D) in the absence or presence of 50 µM MK571. (B, E) Cleavage (activation) of PARP in DLD-1 cells treated for 16 hours with TNF-α (B) or 8 hours with anti-Fas (E) with and without MK571 (C, F). Duplicate experiments for each condition are shown. Caspase-3 activity in DLD-1 cells treated with TNF-α (C) or anti-Fas (F) in combination with different concentrations of MK571. Data are expressed as means ± SD.*

concentrations as low as 6.25 µM (Fig. 3C, F). MK571 (up to 50 µM tested) itself did not induce caspase-3 activity in DLD-1 cells. Taken together, these results show that inhibition of MRP function strongly sensitizes DLD-1 cells for TNF-α- or anti-Fas-induced apoptosis.

Inhibition of MRP1 expression by RNA-interference.

MK571 is a non-specific MRP inhibitor and the different MRPs expressed in DLD-1 cells (Fig. 2B) may contribute to variable extends to cytoprotection. To study the function of MRP1 in this process, we selectively repressed its expression by RNA-

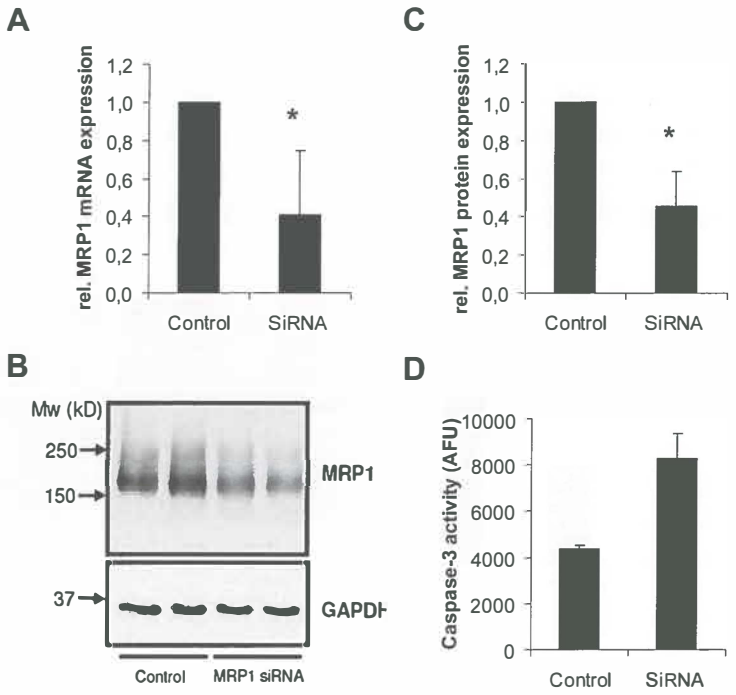

Figure 4. ***Effect of stable overexpression of MRP1 on α-Fas- or TNF-α-induced apoptosis.*** *DLD-1 cells were stably transfected with a plasmid producing MRP1-GFP. Total mRNA was isolated from 2 clones and the native DLD-1 cells. MRP1 mRNA levels were quantified by real time RT-PCR. Compared to DLD-1 cells, MRP1 mRNA levels were increased 17- and 35-fold in DLD1-GFP$^{\#1}$ and DLD1-GFP$^{\#2}$, respectively (A). MRP1 protein expression was determined by Western blotting using specific antibodies against MRP1 (B) and confocal laser scanning microscopy by detecting GFP fluorescence (C). A strong MRP1-specific signal was obtained when analyzing 10 µg of total protein from the recombinant clones. Endogenous MRP1 in DLD-1 cells was only detected when at least 40 µg of total protein extract was analyzed (B). The recombinant MRP1-GFP protein was predominantly present on the plasmamembrane (C, left panel DLD1-GFP$^{\#1}$ and right panel DLD1-GFP$^{\#2}$). MRP1 overexpression resulted in reduced α-Fas-induced caspase-3 activities (D) and PARP-cleavage (E), as well as reduced TNF-α-induced PARP cleavage (F). Caspase-3 activity and PARP-cleavage were determined in total protein extracts of cells exposed for 8 or 16 hours to 1 µg/ml α-Fas and 10 ng/ml TNF-α, respectively. Western blot detection of GAPDH was used as protein loading control.*

interference. DLD-1 cells were transiently transfected with MRP1-specific siRNA duplexes and after 48 hours, MRP1 mRNA (Fig. 4A) and protein (Fig. 4B, C) levels were significantly reduced to approximately 40 % of control cells. Exposure of the siRNA-MRP1 treated DLD-1 cells to anti-Fas led to significantly increased caspase-3 levels (2.0-fold, Fig. 4D). These data show that specific inhibition of MRP1 sensitizes DLD-1 cells to anti-Fas-induced apoptosis and that other MRPs expressed in DLD-1 cells are not able to fully compensate for the loss of MRP1 function.

Stable overexpression of MRP1 in DLD-1 cells.

Next, we generated DLD-1 cell lines that overexpress MRP1 to examine whether this would increase the resistance of these cells against anti-Fas- and TNF-α-induced apoptosis. DLD-1-derivative cell lines, DLD-MRP1-GFP$^{\#1}$ and DLD-MRP1-GFP$^{\#2}$, stably overexpress MRP1 tagged at its C-terminus to the Green Fluorescent Protein

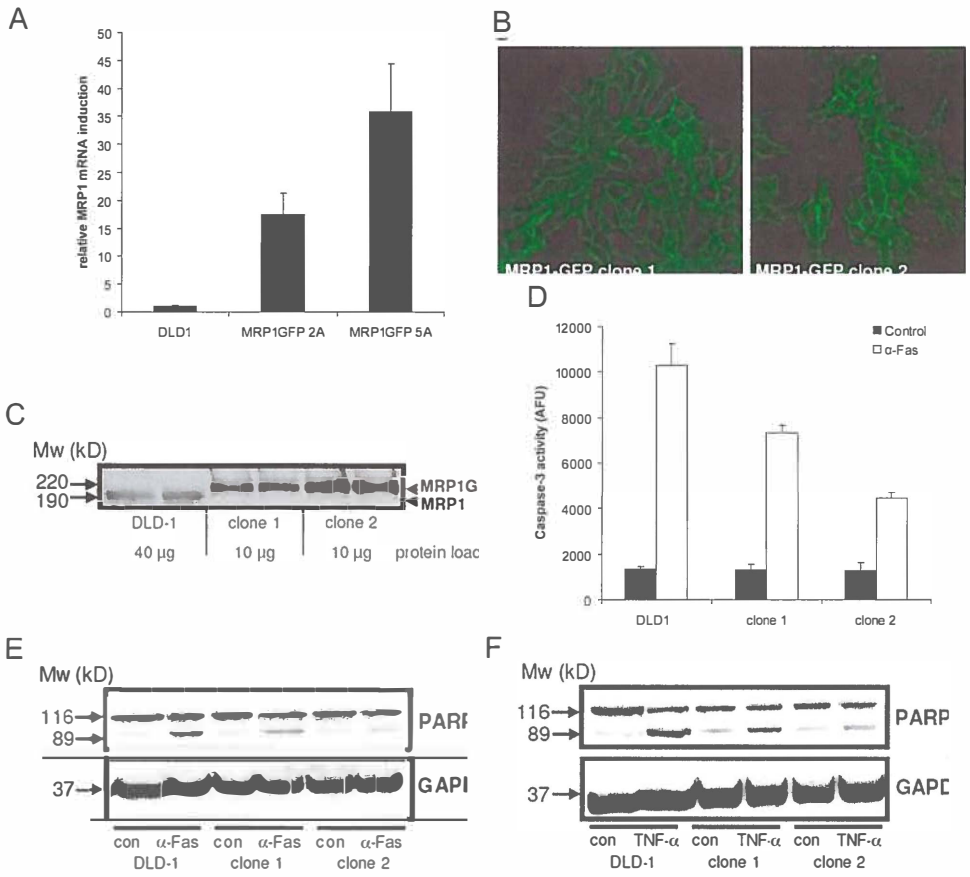

Figure 5. ***Effect of stable overexpression of MRP1 on anti-Fas- or TNF-α-induced apoptosis.*** *MRP1 mRNA (A) and protein (B) expression in DLD-1 cells and two DLD-1-derived cell lines stably transfected with a plasmid producing MRP1-GFP. Western blot analysis revealed the presence of a protein of 220 kDa in both MRP1-GFP-transfected DLD-1-clones, which was detected with the MRP1-specific antibody and had the expected size of GFP-tagged MRP1. Endogenous MRP1 in DLD-1 cells was only detected when at least 40 µg of total protein extract was analyzed. (C) Duplicate experiments for each condition are shown. The recombinant MRP1-GFP protein was predominantly present on the plasmamembrane (DLD1-MRP1-GFP$^{\#1}$ left panel; DLD1-MRP1-GFP$^{\#2}$ right panel). Caspase-3 activity (D) and PARP-cleavage (E, F) in DLD-1 cells and the two DLD-1-MRP1-GFP clones exposed for 8 or 16 hours to 1 µg/ml anti-Fas and 10 ng/ml TNF-α, respectively. Western blot detection of GAPDH was used as protein loading control.*

(MRP1-GFP). Previously, it has been shown that this MRP1-GFP hybrid protein retains its substrate transport activity (32). DLD-MRP1-GFP$^{\#1}$ and DLD-MRP1-GFP$^{\#2}$ contained 17-fold and 35-fold increased MRP1 mRNA levels, respectively (Fig. 5A). Western blot analysis revealed a strong MRP1-specific signal at approximately 220 kDa, the expected size of the hybrid protein consisting of MRP1 (190 kDa) and GFP (30 kDa) (Fig. 5B). In normal DLD-1 cells, only the endogenous MRP1 protein of approximately 190 kDa was detected. Fluorescence microscopical analysis to determine the subcellular location of the GFP signal revealed a predominant plasma membrane staining in the DLD-MRP1-GFP clones (Fig. 5C), which was not detected in the native DLD-1 cells (data not shown). Exposure of the MRP1 overproducing cell lines to anti-Fas or TNF-α resulted in significantly lower caspase-3 activities and PARP-cleavage compared to the native DLD-1 cells (Fig. 5D, E and F). Notably, we observed an inverse correlation between the MRP1-GFP expression level and the level of anti-Fas- or TNF-α-induced apoptosis in these cells. These data imply that MRP1 is able to protect cells from anti-Fas- or TNF-α-induced apoptosis.

Inhibition of cysteinyl leukotriene biosynthesis.

Inflammation induces the conversion of arachidonic acid into cysteinyl leukotrienes. LTC_4 is a high-affinity substrate for MRP1 that is converted to the anti-apoptotic LTD_4 after cellular export. We studied whether blocking MRP function results in the accumulation of a pro-apoptotic compound or, alternatively, prevents the extracellular formation of an anti-apoptotic compound. The lipoxygenase inhibitor AA861 was used to prevent leukotriene synthesis from arachidonic acid. AA861 alone does not induce caspase-3 activity in DLD-1 cells (Fig. 6A). Co-treatment of DLD-1 cells with anti-Fas and increasing amounts of AA861, however, results in a dose-dependent increase in caspase-3 activity. This is probably due to the accumulation of pro-apoptotic arachidonic acid (33;34). Crucial is the observation that the caspase-3 activity in anti-Fas/AA861-treated cells is significantly lower than that in anti-Fas/MK571-treated cells (Fig. 6A). When DLD-1 cells were then co-treated with anti-Fas, MK571 and increasing amounts of AA861, a dose-dependent decrease in caspase-3 activity was observed (Fig. 6A). At the highest concentration AA861 (20 μM), caspase-3 activity in the anti-Fas/MK571/AA861-treated cells was reduced to levels comparable to anti-Fas/AA861-treated DLD-1 cells (Fig. 6A). These data imply that blocking leukotriene synthesis by AA861 results in the accumulation of a pro-apoptotic compound (arachidonic acid) in anti-Fas treated DLD-1 cells. However, AA861 prevents the intracellular accumulation of an even more potent pro-apoptotic compound in anti-Fas/MK571-treated DLD-1 cells, which is a substrate for MRP1

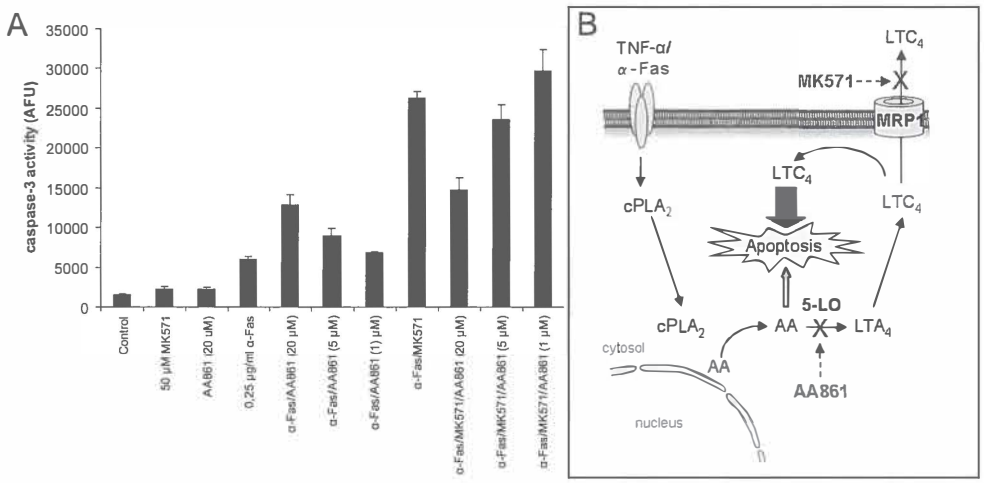

Figure 6. ***Inhibition of leukotriene synthesis in MK-571-treated DLD-1 cells that are exposed to anti-Fas- or TNF-α-induced apoptosis.*** *(A) Caspase-3 activity in DLD-1 cells exposed for 8 hours to 0.25 µg/ml anti-Fas and co-treated with 50 µM MK571 (MRP1 inhibitor) and/or increasing amounts (1, 5 or 20 µM) of AA861 (5-lipoxygenase inhibitor). Data are expressed as means ± SD, * $p<0.05$. (B) Schematic representation of the relationship between MRP1, the leukotriene synthesis pathway and apoptosis. An inflammatory stimulus leads to receptor-mediated intracellular influx of calcium ions. As a consequence, cytosolic phospholipase A_2 ($cPLA_2$) translocates from the cytosol to the nuclear membrane. $cPLA_2$ cleaves arachidonic acid (AA) from the membrane phospholipids. Then, AA is converted by 5-lipoxygenase to leukotriene A_4 (LTA_4), which is converted to LTC_4 through the sequential action of LTB_4 hydrolase and LTC_4 synthase. LTC_4 is exported by MRP1. Inhibition of 5-lipoxygenase leads to the accumulation of pro-apoptotic AA, but the inhibition of MRP1 leads to the accumulation of an even more potent pro-apoptotic agent, LTC_4 (B).*

(Fig. 6B shows a schematic representation of LTC_4 biosynthesis and export and the position and effect of the inhibitors used in this study).

DISCUSSION

In this study, we show that the ABC-transporter MRP1 is induced in inflamed intestinal epithelial cells of patients with inflammatory bowel disease (IBD). Pharmacological inhibition of MRP function increased anti-Fas- and TNF-α-induced apoptosis of DLD-1 cells. Specific reduction of MRP1 expression by RNA interference sensitizes DLD-1 cells for anti-Fas-induced apoptosis. Anti-Fas- and TNF-α-induced apoptosis was reduced in DLD-1 cells stably overexpressing MRP1 or when the intracellular accumulation of its high-affinity substrate, LTC_4, was prevented by inhibition of 5-lipoxygenase.

MRP1 is best known for its ability to protect tumor cells by stimulating efflux of drugs that will otherwise induce apoptosis or necrosis of these cells (35). However, MRP1 is also expressed in normal (non-malignant) tissues such as the colon where it is specifically present in crypt cells that form the proliferative cell compartment of the gut (32;36;37). It has been suggested that this may protect these cells against damage induced by xenobiotics (38). We observed that during intestinal inflammation, MRP1 levels were significantly induced. Using immunohistochemistry, we detected a clear basolateral staining for MRP1 of the crypt cells of the inflamed intestine. The increase in MRP1 expression was not part of a general induction of cytoprotective efflux systems, since the expression of another ABC-transporter MDR1 was decreased under these conditions (11). Therefore, MRP1 may have a specific role in protecting crypt intestinal epithelial cells during inflammation. We provide 4 lines of evidence for such a function of MRP1. Firstly, pharmacological inhibition (by MK571) of MRPs strongly increases cytokine- (TNF-α) and anti-Fas-induced apoptosis in DLD-1 cells. Many earlier studies reported that cytokines or anti-Fas induce apoptosis in intestinal and other cell types/lines (28;29;39;40), but this is the first time that a protective role for an MRP transporter in this process is described. Apparently, exposure to anti-Fas or TNF-α leads to intracellular processes that give rise to apoptotic compounds that are substrates for MRPs. Others have shown that anti-Fas-induced apoptosis is associated with rapid extrusion of reduced glutathione (GSH), which precedes apoptotic cell death (41;42). MRP1 has been suggested to be the transporter responsible for glutathione export under these conditions (43). Blocking MRP1-dependent export of glutathione in anti-Fas treated Jurkat cells led to a decrease in apoptosis, the exact opposite of what we detected for DLD-1 cells. It is relevant to note that controversy exist in recent literature which transporter(s) are responsible for glutathione export in apoptotic cells. Others have suggested that not MRPs, but rather OATPs in jurkat cells are responsible for this effect (44). We further studied the opposite effect of MRP inhibition on anti-Fas-induced apoptosis of T lymphocyte cell lines (Jurkat and MOLT-4) and DLD-1 (Van Steenpaal *et al.*, submitted). We show that cellular glutathione levels are only marginally affected under our experimental conditions and that glutathione depletion is unlikely to be the primary inducting condition for apoptosis in Jurkat and DLD-1 cells.

Secondly, specific repression of MRP1 expression (by RNA interference) sensitizes DLD-1 cells for anti-Fas induced apoptosis. The increased level of apoptosis when expression of specifically MRP1 is reduced shows that, of the MRPs present in DLD-1 cells, MRP1 plays the predominant role in cytoprotection against anti-Fas-

induced apoptosis. Thirdly and in line with the previous observation, recombinant overexpression of MRP1 in DLD-1 cells dose-dependently reduced apoptosis induced by anti-Fas or TNF-α. These data suggest that these compounds induce the intracellular production of an apoptotic compound for which MRP1 is the most important efflux pump. TNF-α and anti-Fas are known to induce apoptosis by activation of membrane receptors (TNF-receptor, Fas) (45). Once activated, these receptors recruit adaptor proteins that initiate a signaling pathway, which ultimately leads to activation of effector caspases that mediate apoptosis. No obvious substrate is present in this signaling pathway that may be exported by MRP1 thereby preventing apoptosis. However, TNF-α or anti-Fas may also induce apoptosis through the release of arachidonic acid from the membrane after activation of Ca^{2+}-dependent cytosolic phospholipases (46). Arachidonic acid stimulates apoptosis through activation of sphingomyelinase and through its downstream metabolites, prostaglandins and leukotrienes. Gastrointestinal epithelial cells have been shown to be able to produce these compounds (47). Indeed and as the fourth line of evidence, we found that blocking the biosynthesis of leukotrienes leads to a reduction in TNF-α- or anti-Fas-induced apoptosis in DLD-1 cells with an inhibited MRP function. Since leukotriene C_4 (LTC_4) is a high affinity substrate of MRP1, we suggest that this is the pro-apoptotic compound during inflammatory conditions (15). MRP4 and MRP6 have also been shown to be able to transport LTC_4 with relatively high affinity(48;49) and both transporters are expressed in human intestinal tissue and DLD-1 cells. However, even a partial reduction of MRP1 in DLD-1 cells already leads to sensitization of these cells for anti-Fas-induced apoptosis. Apparently, MRP4 and/or MRP6 cannot fully compensate for the loss of MRP1 expression.

Collectively, these data show that MRP1 has an important role in protecting the intestinal epithelium during inflammation. Our data correlate well with observations by Nishikawa *et al.* who observed that inhibition of LTC_4 synthesis is associated with decreased intestinal damage induced by trinitrobenzene sulfonic acid-induced colitis (50). In addition, Mrp1 $^{-/-}$ mice have been shown to develop more severe colitis after administration of dextran sulfate sodium (19;50). A cytoprotective role of MRP1 against inflammation-induced cell death may not be restricted to intestinal epithelial cells. Previously, we found that in livers of patients with severe hepatitis, hepatic progenitor cells contain high levels of MRP1 (51). Similar to the intestinal crypt cells, hepatic progenitor cells are involved in regeneration of damaged tissue during pathophysiological conditions. They need to survive in conditions when they are exposed to cytokines, toxic metabolites and xenobiotics. MRP1 may be an important

component of cellular cytoprotection during inflammation.

In conclusion, our data show a novel function of MRP1, that is protection of intestinal epithelial cells against inflammation-induced apoptosis. MRP1 expression is increased in intestinal epithelial cells, in particular those lining the crypt, that may serve to preserve the intestinal regenerative capacity.

Footnotes

The authors thank Dr. S.P. Cole (Queen's University, Kingston, Ontario, Canada) for the kind gift of the pcDNA3.1(-)-MRP1-GFP plasmid and Dr. G.L. Scheffer (VU Medical Center, Amsterdam, The Netherlands) for the mouse monoclonal antibody MRPr1.

The abbreviations used are: iNOS, inducible nitric oxide synthase; ABC-transporters, ATP-binding cassette transporters; IE, intestinal epithelium; MDR1, Multidrug resistance protein 1; MRP, Multidrug Resistance-associated Protein; PARP, poly(ADP-ribose)polymerase

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CHAPTER 3

Mrp1 sensitizes T-helper cells for anti-Fas induced apoptosis; relevance for inflammatory bowel disease

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Abstract

Immune responses are tightly controlled by balancing activation and apoptotic cell death of T-lymphocytes. This process is disturbed in inflammatory bowel diseases (IBD), e.g. Crohn's disease and ulcerative colitis. In particular the resistance of T-lymphocytes to apoptosis poses serious limitations for effective treatment of Crohn's disease. Recently, we found that the Multidrug Resistance-associated Protein 1 (MRP1/ABCC1) protects intestinal epithelial cells against inflammation-induced apoptosis. Here, we studied the role of MRP1 in protecting T-lymphocytes against apoptosis.

MRP1 is highly expressed in peripheral T-lymphocytes and T-lymphocyte cell lines (Jurkat, MOLT-4) compared to monocytes and MRP2, MRP3 and MRP4. MRP1 expression is highly variable (5-fold) in peripheral blood mononuclear cells (PMBCs) of IBD patients compared to PBMCs from healthy volunteers. Pharmacological inhibition of MRP1 decreased anti-FAS-induced apoptosis in Jurkat and MOLT-4 cells, opposite to the effect in intestinal epithelial cells. Modulation of apoptosis in Jurkat and DLD-1 was independent of cellular glutathione levels, which is a substrate of MRP1 and previously implicated in T-cell apoptosis.

These data show that low MRP1 levels in T-lymphocytes lead to resistance to apoptosis through a glutathione-independent mechanism. Induction of MRP1 could therefore be benificial for IBD patients as it protects intestinal epithelium and sensitizes T-lymphocytes for apoptosis.

Introduction

Inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract without an identifiable pathogenic cause (1). Both diseases are characterized by an exaggerated inflammatory response to the endogenous gut flora (2). This leads to increased levels of cytotoxic cytokines in the gastrointestinal tract and eventually to lesions and ulcerations of the intestinal mucosa. Activated T-lymphocytes produce Fas-ligand that, in a feedback mechanism, induces T-lymphocyte apoptosis, thereby controlling immune system homeostasis. In IBD patients, this feedback apoptotic mechanism is disturbed (reviewed in (3)). The hallmark of immunomodulatory drugs used for the treatment of IBD is that they induce apoptosis of T-cells thereby terminating the ongoing inflammation. Unfortunately, this therapy is unsuccessful in a significant number of IBD patients, where T-lymphocytes appear to resist drug-induced apoptosis (4-6). Recent data suggest that ATP-Binding Cassette (ABC)-transporters, and in particular the Multidrug Resistance-associated Protein 1 (MRP1/ ABCC1), play a role in modulating anti-Fas- and cytokine-induced cell death (7,8) In addition, MRP1 is able to export numerous cytotoxic drugs or their metabolites to prevent cell death (9-11).

MRP1 is ubiquitously expressed throughout the body with high expression levels in the brain, kidney, heart, lung and testis. Lower, but considerable expression levels are observed in the intestine and peripheral mononuclear cells (PBMCs) (12-14). MRP1 has a broad substrate specificity, but its function depends strongly on the presence of glutathione (reviewed in (15)). High-affinity endogenous substrates are the pro-inflammatory compound Leukotriene C4 (LTC4) (16), compounds conjugated to glutathione, sulphate or glucuronide and glutathione itself. Although MRP1-mediated multidrug resistance against chemo-therapeutics has been described extensively (reviewed in (78)), the (patho)physiological function of MRP1 is not well understood.

There is increasing evidence that MRP1 has an important function in inflammation and immune responses. Previous studies of our group have shown induction of MRP1 in several inflammatory conditions (18,19). In addition, several mouse studies have shown that absence of Mrp1 result in altered immune responses (20,21). Furthermore, MRP1 induction has recently been identified as an activation marker for human and murine Th-lymphocytes (22-24).

As part of the feedback mechanism to maintain immune system homeostasis, MRP1 has been proposed to sensitize T-cells to anti-Fas induced apoptosis (7,25). Anti-Fas

treatment of Jurkat T-cells was shown to lead to rapid release of reduced glutathione (GSH) via MRP1, which precedes apoptosis. Inhibition of MRP1-mediated GSH export reduced anti-Fas-induced apoptosis.

In a previous study (8), we found increased expression of MRP1 in the inflamed mucosa of IBD patients. Two cell types contained detectable levels of MRP1: intestinal epithelial cells and lamina propria mononuclear cells. This prompted us to study the role of MRP1 in anti-Fas-induced apoptosis in both cell types, since intestinal epithelial cells form the crucial barrier between the antigen-rich gut lumen and the host immune system and mononuclear cells are the target cells for therapy.

In contrast to what has been described for Jurkat T-cells, MRP1 protects intestinal epithelial cells from anti-Fas and cytokine-induced apoptosis (Blokzijl et al, JBC published) by exporting pro-apoptotic compounds from the leukotriene biosynthetic pathway. Since the role of MRP1 in anti-Fas-induced apoptosis of T-cells is currently a matter of debate (7,26,27), we aimed to establish the role of MRP1 in T-cells.

In this study we investigated the MRP1 expression in human T-lymphocytes of healthy volunteers and IBD patients. Next, we compared the MRP expression profile of human T-cells to T-cell lines Jurkat and MOLT-4, as well as the colon carcinoma cell line DLD-1. After establishing the MRP1 transport activity in these cell lines, we determined the effect of several pharmacological inhibitors on anti-Fas induced apoptosis. Finally, we studied whether MRP1-mediated GSH efflux is involved in modulation of apoptosis in these cells.

Materials and methods

Materials

RPE-labeled mouse antibodies to human CD4 and CD8 were obtained from IQP products (Groningen, Netherlands). The monoclonal rat antibody MRPr1 was from Sanbio BV (Uden, Netherlands). Mouse monoclonal antibody GAPDH was from Calbiochem (Darmstadt, Germany). Donkey-anti-rat Alexa 488, culture media, fetal bovine serum, TRIzol reagent and 5-CFDA (5-carboxyfluorescein diacetate) were obtained from Invitrogen Life technologies (Breda, Netherlands). MK571 was purchased from Tebu Bio B.V. (Heerhugowaard, The Netherlands) and oleanolic acid, paraformaldehyde and Triton X-100 were obtained from Sigma-Aldrich chemie B.V. (Zwijndrecht, The Netherlands).

For PBMC isolation, Lymphoprep® was used from Axis-shield (Rodeløkka, Norway).

Penicillin, streptomycin, amphotericin B were from Biowhittaker (Verviers, Belgium)

Fluorescent mounting medium was used from DAKO cytomations (Glostrup, Denmark)

For cell death assays the Annexin apopkit was purchased from Nexins research (Kattedijke, The Netherlands).

Patient characteristics

Blood samples were obtained from healthy controls (n=8), CD patients (n=5) and UC patients (n=4) after informed consent. Disease activity was scored according to Harvey-Bradshaw for CD (29) and a similar index for UC and verified by routine blood values (BSE, Hb, CRP, albumine)

The simplified index for UC is based on 8 variables:

Rectal blood loss:	none (0),	sometimes (1),	frequently (2)
Rectal mucus loss:	none (0),	sometimes (1),	frequently (2)
Stool frequency:	<3 per day (0),	3-6 per day (1),	>6 per day (2)
Stool consistency:	normal (0),	semi liquid (1),	liquid (2)
Painfull instigation:	none (0),	mild (1),	severe (2)
Abdominal pain:	none (0),	mild (1),	severe (2)
Anal pain:	none (0),	mild (1),	severe (2)
Nausea, vomitting:	none (0),	sometimes (1),	frequently (2)

Points for all variables are added and resulting scores is defined as:

0-4 remission, > 4 relapse

The protocol was approved by the Ethics Committee of the University Medical Center Groningen (METc 2002/177c).

Cell culture

The colon carcinoma cell line DLD-1 (DZSM, Braunschweig, Germany) was maintained in RPMI-1640 medium with glutamax supplemented with 10% fetal bovine serum, penicillin, streptomycin and amphotericin B. Cells were passaged twice a

week. Cells were grown to 90% confluency prior to stimulation or RNA isolation. The human leukemia derived T-cell lines Jurkat and MOLT-4 were a kind gift from Dr. L. Visser (dept. Pathology, UMCG). Jurkat and MOLT-4 cells were maintained in RPMI-1640 with glutamax supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (fungizone, 25 ng/ml)(psf). Cells were passaged twice a week. For experiments 500,000 cells were used per condition.

Human peripheral blood mononuclear cells were maintained in RPMI-1640 supplemented with FBS and psf.

Isolation and culture of peripheral blood mononuclear cells

Heparinized blood was diluted one-to-one in sterile PBS after wich it was brought onto a Lymphoprep® density gradient and centrifuged for 20 minutes at 800xg. The mononuclear cell fraction was transferred into a clean tube and washed three times with culture medium. Lymphocytes were purified by attachment of monocytes during two hour culture in a culture flask. Enrichment was checked by quantitative real-time PCR for CD3 expression.

Isolation and quantitative PCR

Total RNA was isolated using the TRI-zol isolation method according to the manufacturers instructions (InVitrogen Life technologies, Breda, Netherlands) Reverse transcription was performed in a final volume of 50 µl. Messenger RNA (mRNA) levels of 18S and MRP1 to MRP4 were quantified using the ABI PRISM 7700 (Applied Biosystems, Calfornia, USA). Real-time PCR conditions were as described previously (19). For quantification of mRNA levels, the CT-value difference was calculated between the gene of interest and the housekeeping gene 18S. This delta Ct was corrected to a linear scale (2-ΔCt) to obtain a relative amount of mRNA compared to 18S. Primer and probe sequences are shown in the supplementary figures.

Western blotting

Total cell lysates were prepared by freeze-thawing cells 4 times in lysis buffer containing DTT, Hepes, PMSF and proteinase inhibitors. Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich,

Germany) using bovine serum albumin as a standard. For MRP1 detection 20 µg of protein was loaded on a 7,5% SDS-PAGE gel. For GAPDH, 5 µg of protein was separated on a 10% SDS-PAGE gel. Protein was transferred by tank blotting. Detection of MRP1 was performed using the primary rat monoclonal antibody Mrpr1 (dilution 1:500) and rabbit-anti-rat peroxidase secondary antibody. GAPDH was detected using mouse monoclonal antibody (dilution 1:5000) antibody and rabbit-anti-mouse peroxidase secondary antibody. The blots were exposed in a ChemiDoc XRS system (Bio-Rad, Hercules, CA) and the intensity of the protein bands were quantified using Quantity One software.

Immunofluorescence microscopy

Cytospins were prepared from Jurkat cells and peripheral blood mononuclear cells (PBMCs). Cytospins were dried overnight at room temperature. The cells were fixed with 4% paraformaldehyde for 20 minutes and subsequently permeabilized using 1% Triton X-100 for 5 minutes. Cytospins were then incubated with the primary antibody overnight at 4°C. Secondary antibodies were incubated for 1 hour at room temperature. Finally, the cytospins were incubated with RPE-labeled antibodies for CD4 or CD8 for 30 minutes at room temperature. Cytospins were then mounted on fluorescence mounting medium and analyzed by confocal microscopy (Leica TCS SP2/AOBS, Rijswijk, Netherland).

5-Carboxyfluorescein (5-CF) efflux assay

MRP1 activity was determined by analyzing the efflux of the fluorescent MRP substrate 5-CF as described by Laupeze et al (12). The cells were loaded for 30 minutes with 1 µM 5-CFDA. Subsequently, cells were washed twice with ice-cold PBS and were resuspended in pre-warmed (37oC) RPMI-1640 (10% FBS, psf) with or without inhibitor. The percentage of 5-CF retained in the cells was determined by flow cytometric analysis on a FACScalibur (BD bioscience) (average fluorescence intensity, AVI). Flow cytometry data was analyzed using Winlist 6.0 software.

The level of MK571- or oleanolic acid-dependent MRP1 inhibition was calculated using the following formulas:

$$\% \text{ 5-CF retained} = ((AVI_{t=20} - AVI_{blanc}) / (AVI_{t=0} - AVI_{blanc})) * 100.$$

% MRP1 inhibition = %5-CF $_{\text{retained MK571/OA}}$ - % 5-CF $_{\text{retained control}}$

Detection of cell death

To quantify apoptosis of DLD-1 cells, caspase-3 enzyme activity was determined 8 hours after stimulation with anti-Fas using a caspase-3 activity kit with fluorimetric detection according to the manufacturer's instructions. (Promega, Leiden, The Netherlands). In Jurkat cells, the percentage of apoptotic cells was determined by flow cytometric detection of Annexin-V and propidium iodide staining after 3 hours of stimulation according to manufacturer's instructions (Annexin apopkit, Nexins research, Kattedijke, The Netherlands). Flow cytometry data was analysed using Winlist 6.0 software. Results are represented as the fold change vs anti-Fas-induced apoptosis. Overlay histograms for illustration were created using WinMDI 2.8 software.

Determination of glutathione concentration

Glutathione concentrations were determined using a spectrophotometric determination as described previously (29). Glutathione concentrations were corrected for protein concentrations.

Statistical analysis

Experiments were performed in triplicate and analyzed by Student's t-test or Kruskall-

Table 1. *Patient characteristics. Included patients were all in clinical remission as determined by the Harvey-Bradshaw score for Crohn's disease and the index for ulcerative colitis as described in materials and methods.*

	patients (male/ female)	Mean Age (years)	Disease score	Medication (with / without)	
Healthy control	8 (5/3)	38,3 (30-43)	n/a	8	n/a
Crohn's disease	5 (3/2)	39,2 (20-56)	0-2	5/0	Azathioprine, 5-aminosalicylic acid, corticosteroids
Ulcerative colitis	4 (1/3)	43,8 (32-55)	0-4	3/1	Azathioprine, 5-aminosalicylic acid

CD score (Harvey-Bradshaw): remission 0-3; relapse >3 UC score: remission 0-4; relapse >4

Wallis and Mann-Whitney tests. All statistical tests were performed in SPSS v12.0 for windows. Significance was defined as $p<0.05$.

Results

MRP1 expression is high in CD4+ Th lymphocytes, with strong variability in IBD patients.

MRP1 expression was detected in epithelial and lamina propria mononuclear cells in the inflamed intestine of IBD patients (9). First, to determine whether MRP1 is differentially expressed in specific mononuclear cells, we analysed its expression

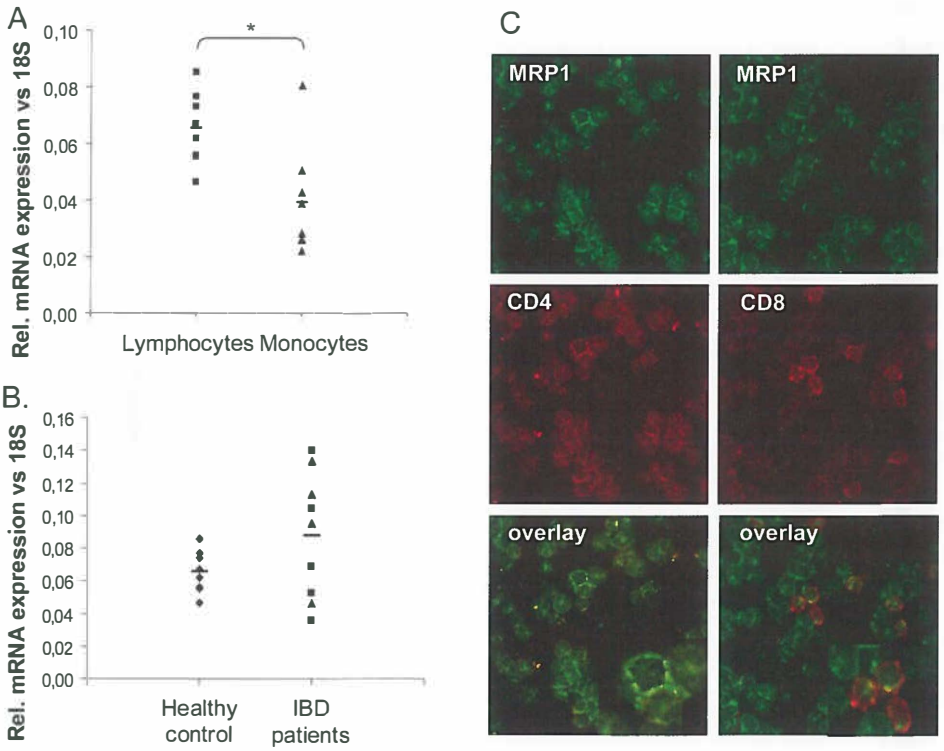

Figure 1. *MRP1 expression in peripheral blood mononuclear cells in healthy control and IBD patients. (A) Peripheral blood mononuclear cells (PBMCs) from healthy controls were subfractionationed into T-lymphocyte- and monocytes/granulocyte-enriched fractions, followed by quantitative PCR to determine MRP1 mRNA levels. * $p < 0.05$ (B) MRP1 mRNA levels in T-lymphocytes from healthy control and IBD patients. Diamonds represent healthy controls, squares represent Crohn's disease and triangles represent ulcerative colitis patients. Real time data are represented as the relative expression versus 18S. (C) Immunofluorescent detection of MRP1 in peripheral blood mononuclear cells and co-staining with CD4 (left panels) or CD8 antibodies (right panels).*

in subfractions of PBMCs. PMBCs were isolated from healthy volunteers and separated into T-lymphocyte- and monocyte/granulocyte-enriched fractions as described in Experimental procedures. RNA was isolated and MRP1 mRNA levels were determined by quantitative RT-PCR fraction (Figure 1A). CD3 mRNA levels were used as marker to determine the enrichment of T-lymphocytes over monocytes/ granulocytes.

Samples in which a 5-fold increase of CD3 expression was found were used for MRP1 detection. MRP1 expression was detected in both T-lymphocytes and monocyte/ granulocyte-enriched fractions with a 1,7-fold higher level in T-lymphocytes. Next, the expression level of MRP1 in lymphocytes from IBD patients in clinical remission were compared to healthy controls. The characteristics of the patients are reported in table 1. A remarkable observation here was the high interindividual variability of lymphocyte MRP1 expression in IBD patients, both in CD and UC, whereas this was not observed for healthy controls. Significantly higher and lower MRP1 levels were detected in IBD patients compared to healthy controls (Figure 1B). However, as a

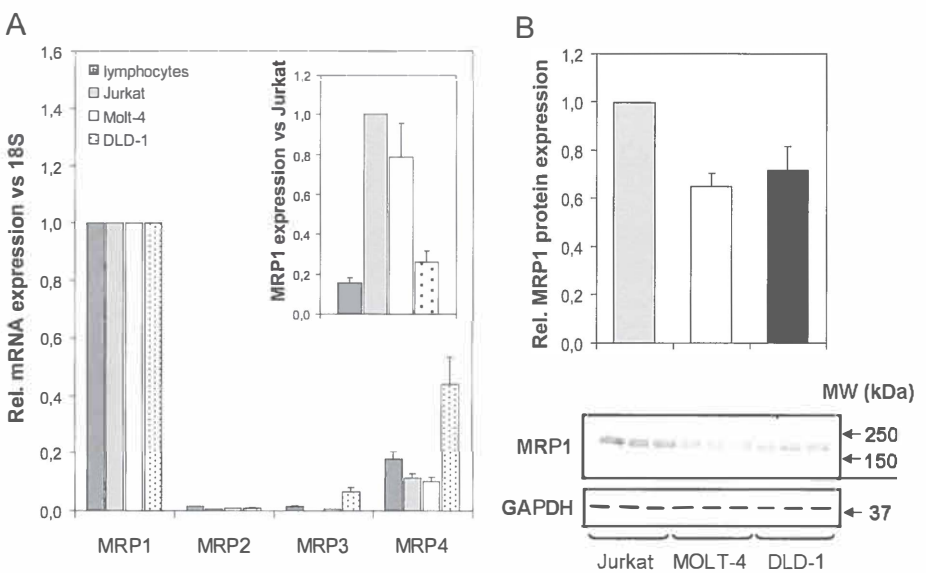

Figure 2. *Expression of MRP1-4 in human lymphocytes, Jurkat, MOLT-4 and DLD-1 cells (A) Expression profile of MRP1-4 in human lymphocytes, Jurkat, MOLT-4 and DLD-1 cells. MRP mRNA expression is correlated to 18S. MRP1 expression in the different cell lines is set to 1. (Inset A) relative MRP1 expression in human lymphocytes, Jurkat, MOLT-4 and DLD-1 cells. MRP1 mRNA expression in Jurkat cells is set to 1 (B) Relative MRP1 protein expression in Jurkat, MOLT-4 and DLD-1 cells. MRP1 protein levels were visualized by Western blotting and protein band intensities were quantified by the Quantity One software and normalized to GAPDH protein levels. Data are expressed as means ± SD*

group, no significant difference was observed in MRP1 expression levels between IBD patients and healthy controls. The MRP1 expression in IBD patients does not correlate to medication or disease score (data not shown).

To determine whether MRP1 expression is present in a subpopulation of lymphocytes, cytospins were prepared from PBMCs isolated from blood from healthy volunteers and co-stained for MRP1 and CD4 (T-helper cells) or CD8 (cytotoxic effector T-cells) (Figure 1C). Confocal laser scanning microscopical analysis revealed that MRP1 shows strong colocalization with CD4. In contrast, no significant colocalisation was detected for MRP1 and CD8 (Figure 1C).

MRP1 is the predominant MRP in primary human T-lymphocytes and human T-cell lines.

To determine whether MRP1 is co-expressed with other MRP family members with overlapping substrate specificity (31,32), we determined the relative mRNA levels of MRP1-4 in human T-lymphocytes (Figure 2A). MRP1 is the most dominant MRP member expressed in these cells. MRP4 expression is at least 5-fold lower compared to MRP1 and MRP2 and MRP3 are almost undetectable in human T-lymphocytes. The MRP expression pattern in human T-lymphocytes is very similar to that observed in Jurkat and MOLT-4 cells, two routinely used model cell lines for T-cells (Figure 2A and (8)). To study the role of MRP1 in T-cells in vitro, we performed experiments with Jurkat cells in a direct comparison to the intestinal epithelial cell line DLD-1. MRP1 mRNA levels are approximately 4-fold lower in DLD-1 cell compared to Jurkat cells. DLD-1 cells further express MRP2-4 in a similar pattern compared to Jurkat cells with MRP1 being the most dominant MRP (Figure 2A).

MRP1 protein expression is subject to post-translational regulation and especially cell-cell contact stabilizes MRP1 protein at the plasma membrane (33,34). We therefore also determined the MRP1 protein levels in Jurkat, MOLT-4 and DLD-1 cells (Figure 2B). MRP1 protein was readily detectable in total protein extracts of these cell types. MRP1 levels were the highest in Jurkat cells with slightly lower levels in MOLT-4 cells mirroring the mRNA levels in these cells. MRP1 protein in DLD-1 and MOLT-4 cells was comparable, even though MRP1 mRNA levels were approximately 3-fold lower in DLD-1 cells. This further substantiates the existence of post-translational mechanisms that control MRP1 protein levels.

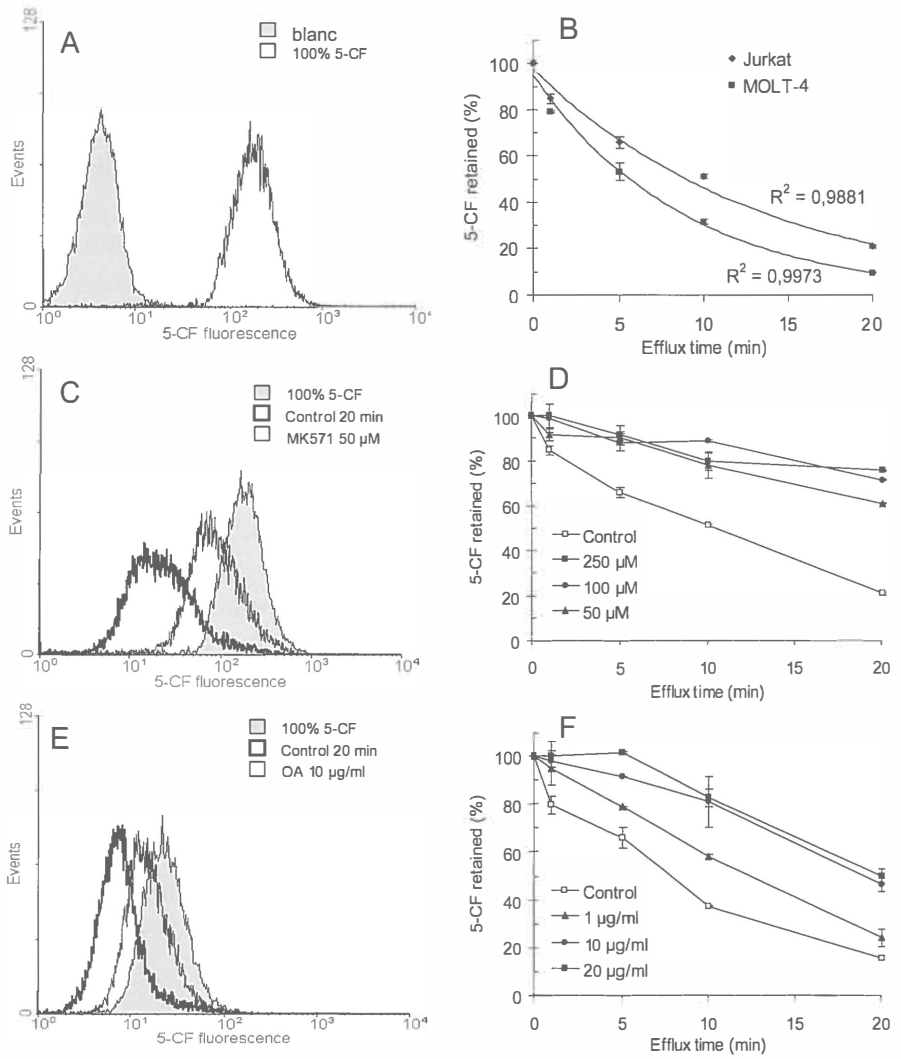

Figure 3. *MRP1 activity in Jurkat and MOLT-4 cells. Cells are loaded with 5-CFDA. (A) Internally, 5-CFDA is converted to the fluorescent MRP1 substrate 5-CF, wich is detected by flow cytometry. Jurkat cells loaded with 5-CFDA (open histogram) and untreated Jurkat cells (filled histogram) are shown. (B) 5-CFDA is washed away and 5-CF efflux is determined in time. The 5-CF fluorescence at t=0 is set to 100%. Triangles represent Jurkat cells, squares represent MOLT-4 cells. MK571 (C,D) and OA (E,F) inhibit 5-CF efflux in Jurkat cells. After 20 minutes of efflux with or without 50 µM MK571 (C) or OA (E), the 5-CF fluorescence is determined. Filled histogram, fluorescence at t=0, open histogram thick line represents fluorescence after 20 minutes of efflux, the open histogram with thin line represents fluorescence after 20 minutes of efflux in the prescence of MK571 or OA. MK571 (D) and OA (F) dose-dependently inhibit 5-CF efflux from Jurkat cells. 5-CF from Jurkat cells was determined in the presence of 0, 50, 100 and 250 µM MK571 (D) or 0, 1 , 10 and 20 µg/ml OA (F).*

MRP function in T lymphocyte and epithelial cell lines

In order to investigate whether MRP1 is located to the plasma membrane and is active in all model cell lines, the export of the MRP substrate 5-carboxyfluorescein (5-CF) was measured. Cells were loaded with 5-CFDA. This compound is taken up by the cells and metabolized intracellularly into the fluorescent MRP substrate 5-CF (figure 3A). Jurkat cells, MOLT-4 (Figure 3D) and DLD-1 cells (data not shown) export 5-CF efficiently. After 20 minutes of export the percentage of 5-CF retained in the cells is 15,7 +/- 4,4 % in Jurkat cells, 23,2 +/- 9,0 % in MOLT-4 cells and 11,2 +/- 0,3 % in DLD-1 cells. Two unrelated pharmacological inhibitors of MRP transport, MK571 and oleanolic acid (OA), were used to inhibit 5-CF export from Jurkat cells (Figure 3 B,C, E and F). MK571 is routinely used at a concentration of 50 µmol/L to block MRP1 dependent export and also in our assay this was sufficient to effectively block export of 5-CF from Jurkat cells (Figure B and E). OA has only recently been identified as an inhibitor of MRP1 transport activity (35,36). We observed a dose-dependent effect of OA and the amount of 5–CF retained in Jurkat cells (Figure 3C and E). At 10 µg/ml a maximal inhibitory effect on the 5-CF efflux was detected.

MK571 is a stronger inhibitor of MRP-mediated efflux of 5-carboxyfluorescein

Next, we determined to what extent these inhibitors inhibit MRP1 in DLD-1, Jurkat and MOLT-4 cells. All cell lines were treated with different concentrations of both MK571 or OA. The highest non-toxic concentrations were used to study the maximal MRP1-inhibition. Both inhibitors (MK571 50 µM; OA 10 µg/ml) increased the amount of 5-CF retained in both Jurkat cells (from 15,7 +/- 4,5% to 50,1 +/- 5,2% and 49,7 +/- 0,5% resp.) and in MOLT-4 cells (from 23,2 +/- 7,8% to 55,7 +/- 4,4% and 58,8

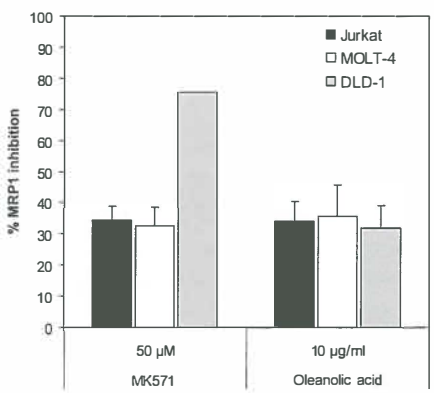

Figure 4. *Inhibition of MRP mediated 5-CF efflux by MK571 or Oleanolic acid in Jurkat, MOLT-4 and DLD-1 cells. 5-CF efflux assays were performed with Jurkat, MOLt-4 and DLD-1 cells in the presence or absence of 50 µM MK571 or 10 µg/ml OA. The % MRP1 inhibition was determined as described in experimental procedures. Data are obtained from 3 individual experiments and are expressed as means ± SD*

+/- 20,9% resp.). The amount of 5-CF retained in DLD-1 cells increased from 11,2 +/- 0,3% in control cells to 86,7 +/- 0,0% and 43,1 +/- 7,1% in cells treated with MK571 or OA, respectively (Figure 4).

MRP inhibition decreases apoptosis in Jurkat cells and increases apoptosis in DLD-1 cells

MRP1 is localized at the plasma membrane and active in all cell lines. Next, to determine the possible role of MRP1 in cell survival, we determined the effect of MRP1 inhibition on anti-Fas-induced apoptosis. Apoptosis was induced in Jurkat and DLD-1 cells by exposure to 1 µg/ml or 0.25 µg/ml anti-Fas, respectively, in the presence or absence of the MRP inhibitors MK571 (50 µmol/L) or OA (10 µg/ml) (Figure 5). Anti-Fas induces apoptosis in both Jurkat (3.3-fold induction vs control) and DLD-1 cells (12.3-fold induction vs control). Co-treatment of Jurkat cells with anti-Fas and 50 µmol/L MK571 or 10 µg/ml OA resulted in a significant reduction of the percentage of apoptotic cells (-45% and -55%, respectively) compared to anti-Fas treatment alone (Figure 5A). In contrast, co-treatment of DLD-1 cells with anti-Fas and MK571 or OA significantly induced the caspase-3 activity 2.3- or 2.4-fold, respectively, compared to anti-Fas treatment alone (Figure 5B). Treatment of Jurkat or DLD-1 cells with only MK571 or OA did not induce apoptosis.

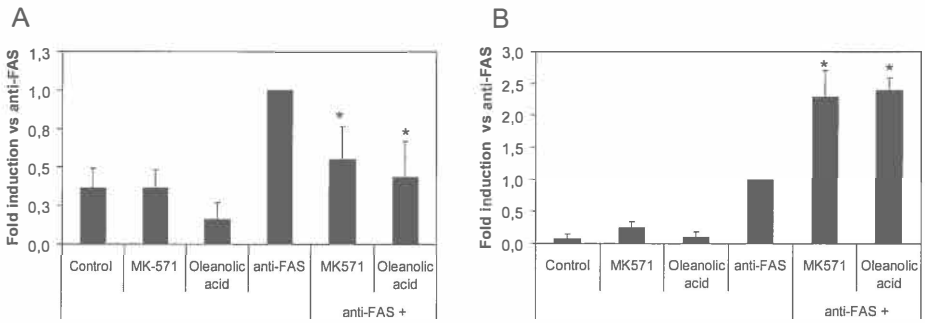

Figure 5. *MRP inhibition differentially modulates apoptosis in Jurkat and DLD-1 cells. Apoptosis was induced by stimulation with 1 µg/ml anti-FAS for 3 hours in Jurkat cells (A) and 0.25 µg/ml for 8 hours in DLD-1 cells (B). (A) Inhibition with both 50 µM MK571 or 10 µg/ml OA reduces the number of anti-FAS-induced apoptotic Jurkat cells determined by Annexin-V/propidium iodide staining. (B) Treatment of MK571 or OA induces anti-FAS-induced apoptosis in DLD-1 cells determined by caspase-3 activity. MK571 and Oleanolic acid alone do not induce apoptosis. Anti-FAS-induced apoptosis is set to 1. Data are obtained from 3 individual experiments and are expressed as means ± SD (* $p < 0.05$ vs anti-FAS)*

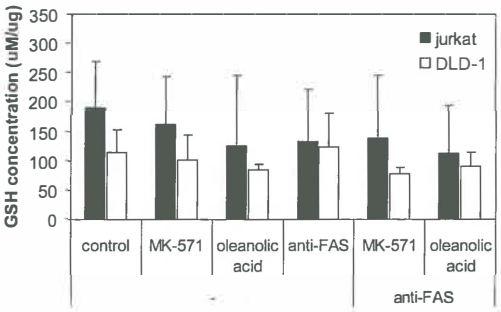

Figure 6. *Glutathione concentration in Jurkat and DLD-1 cells after anti-FAS-induced apoptosis.. Jurkat and DLD-1 cells were treated with 50 µM MK571, 10 µg/ml OA, anti-FAS (0.25 µg/ml for Jurkat; 1 µg/ml for DLD-1) and combinations, followed by determination of the total intracellular glutathione concentrations.*

Increased survival of T cells by MRP1 inhibition is not a result of inhibition of glutathione export.

MRP1-dependent export of reduced glutathione (GSH) has previously been proposed to aid to anti-Fas induced apoptosis of Jurkat cells (8) We determined the cellular GSH concentrations at the peak of apoptosis in anti-Fas-treated Jurkat (3 h) and DLD-1 cells (8 h). Untreated Jurkat and DLD-1 cells contain cellular GSH concentrations of 190 +/-78 and 114 +/-39 nM/µg protein, respectively. In Jurkat cells, anti-Fas alone, or in combination with MK571 or OA, did not significantly change the cellular GSH concentration (Figure 6A). Similarly, GSH levels in anti-Fas- or MK571-treated DLD-1 cells were comparable to untreated control. Remarkably, OA treatment alone leads to a decrease in cellular GSH content (-25% versus control), but did not induce apoptosis. DLD-1 cells co-treated with anti-Fas and OA or MK571 show strongly increased caspase-3 activities (Figure 5), but cellular GSH levels were comparable to anti-Fas-treated DLD-1 cells. These data show that anti-Fas-dependent modulation of apoptosis in Jurkat and DLD-1 cells is independent of cellular GSH levels.

Discussion

The multidrug resistance-associated protein 1 (MRP1) is strongly expressed in 1) mononuclear cells and 2) epithelial cells in the inflamed intestine of patients with IBD. We show that MRP1 has opposite roles in modulating anti-Fas-induced apoptosis in mononuclear cells and epithelial cells (chapter2 MRP1 sensitizes Jurkat cells for anti-Fas-induced apoptosis, while protecting intestinal epithelial DLD-1 cells. This opposite effect is particularly relevant for IBD, since currrent therapies are aimed at inducing apoptosis in T-cells and recovery of the intestinal epithelium.

Apoptosis resistance of T-lymphocytes is a common phenomenon in IBD patients, in particular in Crohn's disease patients, as it contributes to the development of the disease and negatively affects medical treatment.

To investigate the function of MRP1 in the apoptotic response of human T-lymphocytes we used Jurkat cells as a model cell type. The expression profile of MRPs with an overlapping substrate specificity (MRP1 to MRP4) is comparable between human T-lymphocytes and Jurkat cells, with MRP1 being the most dominantly expressed. This validates the choice of Jurkat cells as a model T-cell line for studying the role of T-cell MRPs, and MRP1 in particular.

We found that pharmacological inhibition of MRP function using several MRP1 inhibitors strongly decreased anti-Fas-induced apoptosis in Jurkat cells, whereas it increased anti-Fas induced apoptosis in DLD-1 cells. The latter observation correlates to the results from our previous study (6). MRP1-mediated protection in DLD-1 cells was shown to be due to the export of a pro-apoptotic component from the leukotriene biosynthesis pathway. In the present study, we also tested whether MRP1-dependent export of leukotriene (metabolite)s may be involved in sensitizing Jurkat cells to apoptosis. Inhibition of leukotriene synthesis by AA861, either in the presence or absence of MRP1-inhibition, did not affect the level of apoptosis (data not shown). Therefore, we conclude that such leukotriene(metabolite)s are not involved in the MRP1-mediated sensitization of Jurkat cells to anti-Fas induced apoptosis.

Recently, controversial observations were made regarding the possible role of MRP1 in anti-Fas-induced glutathione efflux from Jurkat cells, which may be directly linked to induction of apoptosis. Franco et al. and Hammond et al. both showed that anti-Fas treatment of Jurkat cells leads to cellular glutathione efflux (7,26). However, opposite effects of MK571 treatment were observed. Hammond et al. showed that MK571 treatment inhibited the glutathione efflux and decreased anti-Fas induced apoptosis. Specific inhibition of MRP1 by RNA interference also reduced the anti-Fas induced glutathione efflux. They therefore suggested that MRP1 inhibition protects Jurkat cells by reducing glutathione efflux. In contrast, Franco et al observed that MK571 actually increased anti-Fas-induced glutathione efflux from Jurkat cells, leading to a further increase in apoptosis (36). As a consequence, they propose that glutathione efflux is not mediated by MRP1, but rather by SLC0/OATP-type transporters. To complicate matters even further, Hentze et al. found that glutathione depletion in fact inhibits anti-Fas-induced apoptosis in several B- and T-lymphoblastoid cell lines including Jurkat cells (37). These contradictory results may, in part, be a result of different experimental approaches used. Hammond et al

present the relative distribution of GSH between cells and the medium after anti-Fas treatment. While the relative amount of extracellular GSH increases after anti-Fas treatment, the absolute amount of cellular GSH remained unclear. In addition, all measurements by Hammond et al were performed in glucose-depleted conditions, which might influence the apoptotic response or even induce cell death. Franco et al. only determined the intracellular pools of GSH by flow cytometry. In these studies, monochlorobimane is added to the cells, that after diffusion into the cells, is conjugated to GSH to form fluorescent bimane-glutathione. This conjugate, however, is a substrate for MRP1 (38). This complicates the interpretation of these results as the cellular levels of bimane-glutathione depend on 1) monochlorobimane diffusion rate, 2) GSH conjugation rate by gluthation-S-transferases and 3) MRP-dependent export of bimane-glutathione. In our studies, we maintained the Jurkat and DLD-1 cells in normal growth conditions to observe the most natural reaction to anti-Fas treatment. In addition, we measured the absolute intracellular glutathione concentration after cell lysis using a spectrophotometric assay. We found no decrease in intracellular glutathione concentration during anti-Fas-induced apoptosis in Jurkat cells. Glutathione concentrations were also not significantly altered when cells were co-treated with anti-Fas and MK571 or oleanolic acid. Oleanolic acid treatment alone, however, did show a trend towards decreasing intracellular glutathione concentrations which correlates to the transactivation of GSH efflux by MK571 treatment, as observed by Franco et al.

Since apoptosis is an essential process in the removal of activated T-cells and therefore important in the regulation of the immune response (reviewed in (3)), failure to express MRP1 or low MRP1 expression could lead to apoptosis-resistant T-lymphocytes. In this respect, it is relevant to note that MRP1 expression in lymphocytes from IBD patients in clinically non-active disease showed strong variation. In contrast, MRP1 levels in T-lymphocytes from healthy controls were highly comparable between individuals. However, for the IBD patients included in this study, MRP1 expression levels did not correlated to either the disease score or specific medication.

In this study, we show that MRP1 is expressed exclusively in CD4-positive T cells compared to CD8-positive T cells. Previous studies have indicated that MRP1 expression in CD4 positive T-cells is increased during activation and in addition, that MRP1 inhibition alters the cytokine profile. Since CD4-positive T-cells produce (apoptotic) cytokines during inflammation, the MRP1 expression could have several functions. It might be involved in the export of the cytokines from the cell and/or it

provides a mechanism for apoptosis to regulate the inflammatory response.

Our previous studies indicate a cytoprotective function of MRP1 in intestinal epithelial cells. These two studies combined indicate a dual role for MRP1 in modulating apoptosis, which is particularly relevant during intestinal inflammation. Increased levels of MRP1 in the intestinal epihelium reduces tissue damage via anti-Fas-induced apoptosis, whereas the important target of IBD-therapy, the T-lymphocytes, become more sensitive to apoptosis. Upregulation of intestinal MRP1 expression could therefore, be considered as a treatment option for patients with active inflammatory bowel disease. However, further studies to investigate the clinical application are necessary.

Acknowledgements

The authors thank the volunteers and patients for participating in this study.

The kind gift of the Jurkat an MOLT-4 cell lines by Dr. Visser from the department of Pathology (University Medical Center Groningen, Groningen, The Netherlands) is greatly appreciated

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CHAPTER 4

Mrp1 is not required for hepatocyte- and hepatic progenitor cell-dependent liver regeneration in mice.

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Abstract

Hepatic progenitor cells (HPCs) and bone marrow stem cells contribute to liver regeneration when hepatocyte proliferation is compromised, e.g. during severe liver injury. The multidrug resistance-associated protein 1 (MRP1) is expressed in HPCs as well as in bone marrow nuclear cells (BMNCs). Recently, we found that MRP1 provides cellular protection against cytokine-induced apoptosis. Induced expression of MRP1 was observed in the regenerative compartment of both the intestinal epithelium in the inflamed mucosa of patients with inflammatory bowel disease and the liver of patients with diseases associated with hepatic inflammation. Thus, MRP1 may protect stem cells to allow tissue regeneration in pathophysiological conditions. The aim of this study was to determine whether Mrp1 is required for stem cell-dependent liver regeneration in mice.

Mrp1-/- mice and wild type control mice were subjected to 70% partial hepatectomy (pHx) in the presence or absence of 2-acetylaminofluorene (2-AAF) to block hepatocyte proliferation. Liver regeneration was monitored by liver weight recovery. Hepatic expression of genes encoding Mrp's and stem cell markers (alpha-fetoprotein-AFP) was determined by quantitative PCR. Cell proliferation was analyzed by immunohistochemical microscopy after Ki-67 staining.

Three days after 70% pHx, liver weight in wild type and Mrp1-/- mice was 85% of the pre-operative liver mass, independent of the 2-AAF treatment. 2-AAF treatment lead to a strongly increased AFP expression in PHx-treated wild type mice, which was not observed in Mrp1-/- mice. 2-AAF treatment increased the number of Ki-67 positive cells to a similar level in the livers of pHx-treated wild type and Mrp1-/- mice. Mrp3 expression was significantly increased in untreated Mrp1-/- mice compared to wild type controls., but was comparable in the 2-AAF/pHx-treated groups.

Stem cell activation is compromised in Mrp1-/- mice, but does not affect liver regeneration in 2-AAF-treated animals.

Introduction

The liver plays an important role in glucose, fat and vitamin metabolism as well as in detoxification and in immune responses. Liver diseases are therefore associated with a variety of symptoms. For most functions, the liver has a significant overcapacity. Symptoms demonstrate only when the liver suffers from major injury. Liver disease is potentially reversible as the liver has the capacity to regenerate after loss of functional tissue. This maintains liver function and repair during diseases such as liver fibrosis, viral hepatitis or alcohol and drug toxicity (1). Moreover, it allows the regrowth of the liver after surgical procedures such as tumor resection or liver transplantation.

Cell renewal in the healthy liver is very limited. Hepatocytes are the functional liver cells and make up over 80% of the liver. Under normal conditions, only 1 in 20.000-40.000 hepatocytes divides. However, when liver mass is lost, hepatocytes re-enter the cell cycle and start to proliferate until the liver is restored to its original size. This is a very efficient and fast process when no complicating factors are present. Removal of 70% of the liver in mice or rats is fully compensated in 7-10 days in which the liver regrows to its original size (2). In many liver diseases, however, hepatocyte proliferation is compromised due to the increased levels of cytotoxic factors, such as cytokines, bile salts and reactive oxygen species (ROS). Liver regeneration can still occur under such conditions through the activation, proliferation and differentiation of liver- and/or bone marrow-derived stem cells. Liver-specific stem cells, also called hepatic progenitor cells (HPCs) or oval cells, are bipotent cells that can differentiate into functional hepatocytes and cholangiocytes (bile canaliculi epithelial cells) (3,4) and are located in the canals of Hering where the bile canaliculi merge into interlobular bile ducts. Activation of HPCs/oval cells is morphologically detected as ductular reactions. Liver damage has also been shown to induce hepatic gene expression profiles in bone marrow cells (5) and mesenchymal stem cells (MSCs) and hematopoietic stem cells can transdifferentiate to hepatic cell lineages in vitro and repopulate the damaged liver in vivo (recent reviews by (6-8))

Our earlier studies have shown the induction of specific multidrug resistance-type ATP-binding cassette (ABC) transporters in HPCs in regenerating rat liver, as well as in livers from patients with primary biliary cirrhosis and chronic viral hepatitis C (9,10). Specifically, multidrug resistance protein 1 (MDR1/P-glycoprotein), multidrug resistance-associated protein 1 (MRP1) and MRP3 were strongly induced. These proteins have been shown to protect (cancer) cells against a wide variety of structurally unrelated drugs and endogenous metabolites. Of particular interest is the induction of MRP1, which transports phase-2 metabolites (glutathione-, sulphate-

and glucuronide-conjugates)(11), glutathione (12) and the pro-inflammatory cytokine leukotriene C4 (LTC4) (13). In the injured liver, MRP1 is expressed in HPCs as well as in activated hepatic stellate cells that cause fibrosis (14) The level of MRP1 in hepatocytes is low. Notably, MRP1 is also significantly expressed in Sca+ and/ or c-kit+ mouse HSCs (15). The presence of MRP1 in stem cells and its induction in liver disease suggest that this protein may have additional functions, besides its well-known drug transporting activity. Recently, we found that MRP1 provides cytoprotection to intestinal epithelial cells against cytokine-induced apoptosis (16). MRP1 expression was specifically detected in the regenerative compartment of the intestinal epithelium. Similarly, MRP1 may protect HSCs and HPCs against cytotoxic factors that accumulate during liver disease so that the liver can regenerate from these cells.

In this study, we analyzed the functional relevance of Mrp1 during liver regeneration in mice, specifically under conditions where hepatocyte proliferation is blocked. Wild type and Mrp1-/- mice were subjected to 70% partial hepatectomy in the absence and presence of 2-acetylaminofluorene (2-AAF), which selectively inhibits hepatocyte proliferation (5,17)

Materials and methods

Animals

Five to eight week-old Mrp1-/- (FVB.129P2-Abcc1atm1Bor N1) mice and their wild-type counterparts (FVB/Ntac) were obtained from Taconic farms (Denmark). Animals were kept in a 12 hour light/dark regime and were fed standard chow and water ad libitum. The animal experiments were approved by the ethics committee of the medical faculty of the University of Groningen (D4757).

2-AAF treatment and partial hepatectomy

2-Acetylaminofluorene (2-AAF, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) was dissolved in DMSO (200 mg/ml, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Mice were injected with 30 mg/kg 2-AAF (WT, n=10; Mrp1-/-, n=10) or vehicle (corn oil/5% DMSO, Sigma-Aldrich Chemie B.V.) (WT, n=10; Mrp1-/-, n=10) daily for 7 days. Subsequently, sham or partial hepatectomy (pHx) was performed as described (18), generating 8 treatment groups of n=5. Briefly, animals were anaesthetized using isoflurane. The abdomen was opened and

a suture was applied proximal to the liver lobe. The lobe was removed as close as possible to the suture. This process was performed for all anterior lobes, resulting in ± 70% pHx. A final dose of 2-AAF or vehicle was administered into the peritoneal cavity, followed by closure of peritoneum and skin. 2,5 ml of saline (37^{0}C), was injected subcutaneously to compensate for fluid loss during the surgery. Animals were kept in a recovery incubator during the first night after pHx or sham operation. Buprenorphine (0,05 mg/kg) was administered subcutaneously 12 h and 24 h after surgery. The animals were sacrificed 72 hours after surgery. The liver was removed, weighted and samples were stored in liquid nitrogen or fixed in 4% formaldehyde.

AST/ALT determination in blood

Blood was stored in EDTA-containing tubes (Greiner Bio-one, Kremsmunster, Austria). Plasma was obtained by centrifugating the blood samples for 15 minutes at 10,000 rpm. AST and ALT enzyme concentrations were determined using the Spinreact GOT and spinreact GPT kit according to the manufacturer's instructions (Spinreact, Girona, Spain).

RNA isolation and quantitative PCR

Total RNA was isolated using the TRIzol isolation method (InVitrogen Life Technologies, Breda, The Netherlands) according to the manufacturers instructions. cDNA was synthesized in a final volume of 50 µl as described before (16). Messenger RNA (mRNA) levels of 18S, Mrp1 to Mrp7 and alpha-fetoprotein (AFP) were quantified using the ABI PRISM 7700 (Applied Biosystems, California, USA). Real-time conditions were as previously described (10). For quantification of mRNA levels, the difference was calculated between the gene of interest and the housekeeping gene 18S. This delta Ct was corrected to a linear scale (2-ΔCt) to obtain a relative amount of mRNA compared to 18S. Real time primers and probe sequences are shown in Table 1.

Immunohistochemistry

Immunohistochemistry for Ki-67 was performed on 4 µm-thick slices of paraffin embedded tissues. Antigen retrieval was performed using 10mM citrate buffer (pH 6,0). Slides were stained with the rat anti-mouse Ki-67 monoclonal antibody (dilution1:50;

Table 1: *realtime Primers and probes*

Gene	Primer/Probe	Sequence
18S	Forward	5'-CGGCTACCACATCCAAGGA-3'
	Reverse	5'-CCAATTACAGGGCCTCGAAA-3'
	Probe	5' FAM-CGCGCAAATTACCCACTCCCGA-TAMRA 3'
Mrp1	Forward	5'-TGAAACAGAGAAGGAGGCTCCT'-3'
	Reverse	5'-AGGCAGTAATCCCGGAACTCTA-3'
	Probe	5' FAM-TGGCCCCATTCAGGCCGTG-TAMRA 3'
Mrp2	Forward	5'-GGATGGTGACTGTGGGCTGAT'-3'
	Reverse	5'-GGCTGTTCTCCCTTCTCATGG-3'
	Probe	5' FAM-AGCTGCATCGTCAGGAATTTCCTCCACA-TAMRA3'
Mrp3	Forward	5'-TCCCACTTTTCGGAGACAGTAAC-3'
	Reverse	5'-ACTGAGGACCTTGAAGTCTTGGA-3'
	Probe	5' FAM-CACCAGTGTCATTCGGGCCTATGG C-TAMRA 3'
Mrp4	Forward	5'-GCCGACATCTACCTCCTTGAT G-3'
	Reverse	5'-CGTGCAACGCCTGACAGA-3'
	Probe	5' FAM-CCCACTTCTGCATCGACAGCGCT-TAMRA 3'
Mrp5	Forward	5'-CGGAGAACAAGATCGTTGGAAT-3'
	Reverse	5'-CAGGGAAAGCCCCTCAACTC-3'
	Probe	5' FAM-CCAAGATGCTCTCGAAACAGCAGCCC-TAMRA 3'
Mrp6	Forward	5'-CCACAGGATTGACAGCAGAAGA-3'
	Reverse	5'-CGCAGGTAGCTCAGGTATATGGT-3'
	Probe	5' FAM-TCTTCACCCGGCCATATCGCACAC-TAMRA 3'
Mrp7	Forward	5'-GGGGCCACTTACAGGTTTGA-3'
	Reverse	5'-ATCGTGGCATAGGAAGCAAACT-3'
	Probe	5' FAM-AACCAGCGACTCTTGGAGCTGAACCA-TAMRA 3'
AFP	Forward	5'-GCTCAGTACGACAAGGTCGTTCT-3'
	Reverse	5'-TGGATGCTCTCTTTGTCTGGAA-3'
	Probe	5' FAM-TCCTCCTTGTTGTCAGCTTTGCAGCA-TAMRA 3'
Mdr1a	Forward	5'-GCAGGTTGGCTAGACAGGTTGT-3'
	Reverse	5'-GAGCGCCACTCCATGGATAA-3'
	Probe	5' FAM-AGCAGCCAGAGTTCCCACCAGCATG-TAMRA 3'
Mdr1b	Forward	5'-GCTGGACAAGCTGTGCATGA-3'
	Reverse	5'-TGGCAGAATACTGGCTTCTGCT-3'
	Probe	5' FAM-CTTCCCCTCTTGATGCTGGTGTTTGGAAAC-TAMRA 3'
Mdr2	Forward	5'-GCAGCGAGAAACGGAACAG-3'
	Reverse	5'-GGTTGCTGATGCTGCCTAGTT-3'
	Probe	5' FAM-AAAGTCGCCGTCTAGGCGGCGCCGT-TAMRA 3'

Dako Cytomation, Denmark A/S Glostrup), the secondary antibody polyclonal rabbit anti-rat immunoglobulin/HRP (dilution 1:50; Dako Cytomation, Denmark A/S Glostrup) and tertiary antibody polyclonal goat anti-rabbit immunoglobulins/HRP (dilution 1:50; Dako Cytomation, Denmark A/S Glostrup), followed by 10 minute incubation in DAB-chromogene (Dako Cytomation, Denmark A/S Glostrup) and haematoxylin counterstaining.

Statistical analysis

Experiments were performed in triplicate and analyzed by Student's t-test or Kruskall-Wallis and Mann-Whitney tests. All statistical tests were performed in SPSS v14.0 for Windows. Significance was defined as $p<0,05$.

Results

Compensatory Mrp expression profile in Mrp1 knockout mice

We first determined the relative mRNA levels of Mdr1a, 1b, 2 and Mrp1-7 in the livers of wild type and Mrp1-/- mice to identify putative compensatory mechanisms induced by the absence of Mrp1 (Figure 1). Mrp2 is the most dominantly expressed Mrp in the liver of wild-type mice. Significant levels of Mdr2, Mrp3, 6 and 7 mRNAs are also detected, but are substantially lower than Mrp2. Mrp1, Mrp4, Mrp5, Mdr1a and Mdr1b expression is low. The absence of Mrp1 does not result in strong changes in the hepatic Mdr/Mrp expression profile, except for Mrp3, which is significantly increased (1.9-fold) compared to wild type mice. In addition, the low levels of Mdr1b in wild type mice are further decreased in Mrp1-/- mice.

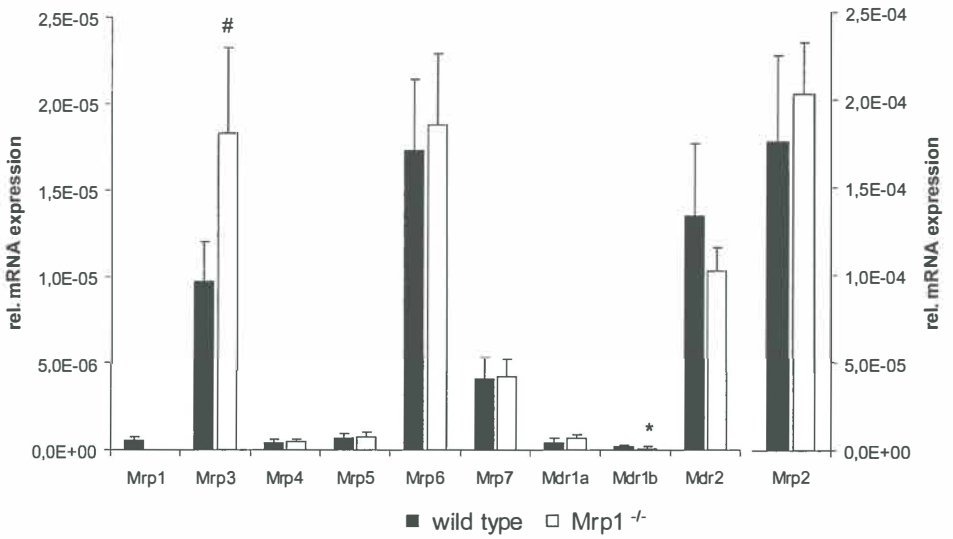

Figure 1. *Mrp expression profile in wild type and Mrp1-knockout mice. Relative mRNA expression was determined by Q-RT PCR in liver of vehicle/sham treated mice (both wild type and Mrp1-/-). Expression was normalized to 18S expression and represented as the relative mRNA expression (2^-ΔCt). Data represent means + SD (n=5). * p < 0,05 vs wild type. # p < 0,01 vs. wild type. Significance was determined by Mann-Whitney test.*

Bodyweight recovery after partial hepatectomy is not influenced by 2-AAF treatment or the absence of Mrp1.

The dynamics of liver regeneration in the presence of 2-AAF (hepatocyte proliferation blocker) and/or the absence of Mrp1 has not been studied before in mice. Therefore, we next determined whether mice survived 70% pHx in combination with 2-AAF treatment and/or Mrp1-deficiency. Bodyweight of the animals was monitored after surgery. Both wild type and Mrp1-/- mice survived combined 2-AAF/pHx-treatment at least up to day 3 after surgery. Sham operation caused a comparable weight loss in wild type and Mrp1-/- mice of approximately 5% at day one, after which the body weight stabilized (Figure 2A and B). Wild-type mice that underwent pHx lost more weight (corrected for explant weight) compared to sham operated mice. This difference was significant at day two after surgery both for vehicle-treated (sham 93.4% +/- 1.9 and pHx 87.5% +/- 2.3) as well as 2-AAF-treated mice (sham 92.2% +/- 2.3 and pHx 88.7% +/- 1.4). At day 3, pHx-treated wild-type mice had regained weight and the bodyweight was similar to sham-operated animals, both in the vehicle- and 2-AAF-treated group.

Partially hepatectomized Mrp1-/- mice also showed a trend towards increased

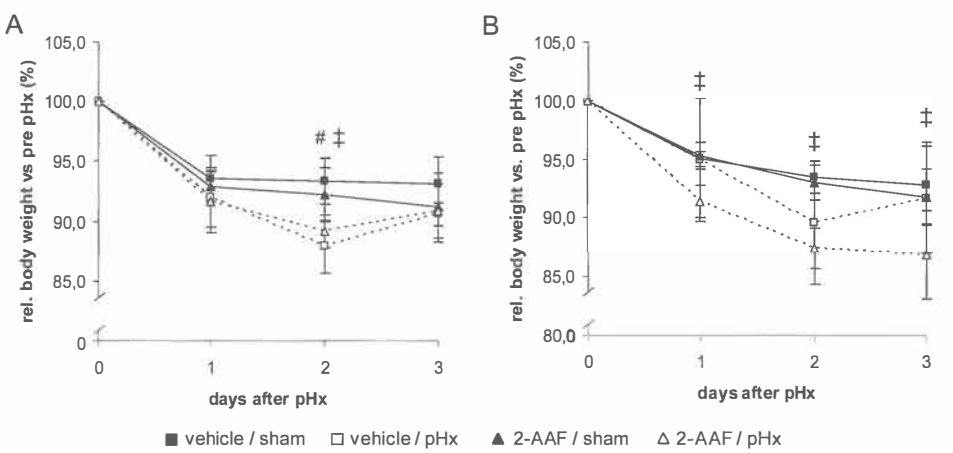

Figure 2. *Body weight of mice after sham or hepatectomy operation. Mice were weighed before and daily after sham operation or hepatectomy. Body weight before partial hepatectomy was set to 100%. The weight was corrected for the mass of the removed liver tissue. The relative body weight was determined daily for both wild type (A) and Mrp1-/- (B) mice. Data represent means +/- sd (n=5). # p<0,05 vehicle/sham vs. vehicle/pHx; ‡ p<0,05 2-AAF/sham vs. 2-AAF/pHx. Significance was determined by Mann-Whitney test.*

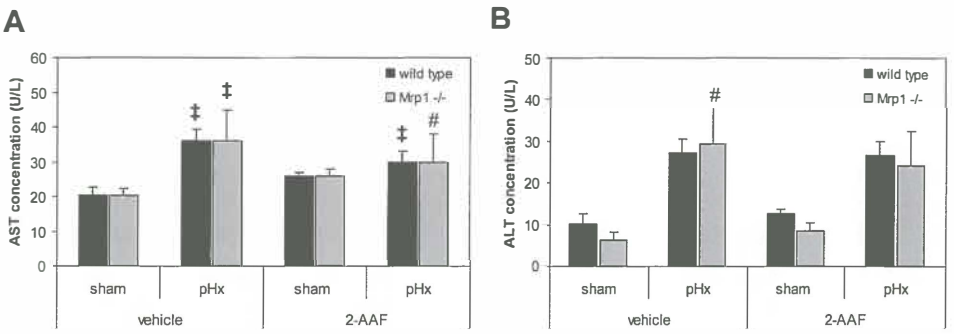

Figure 3. *AST and ALT concentrations in blood 3 days after pHx. To analyze liver damage, ALT and AST levels in blood were determined. Data show means + sd (n=5, except WT 2-AAF/sham n=4). # p < 0,05 vs. sham treated group. ‡ p < 0,01 vs. sham treated group. Statistics determined by T-test.*

bodyweight loss at day 2, however, the difference to sham-operated Mrp1-/- mice was not significant (p=0.70) (Figure 2B). In contrast, 2-AAF/pHx-treated Mrp1-/- mice lost significantly more body weight compared to sham- (p=0.001 at day 2) or 2-AAF- (p=0.01 at day 2) treated animals. In contrast to 2-AAF/pHx-treated wild type mice, the body weight of the 2-AAF/pHx-treated Mrp1-/- mice did not recover at day 3. These data suggest that Mrp1 is required for post-pHx recovery of mice when hepatocyte proliferation is blocked.

The absence of Mrp1 does not increase liver damage after pHx.

Serum aspartate transferase (AST) and alanine transferase (ALT) levels were analyzed at 3 days after pHx to determine whether the absence of weight gain in the 2-AAF/pHx-treated Mrp1-/- mice was associated with increased liver damage (Figure 3). A minor, but significant increase in AST levels was detected in pHx-treated mice, which was similar for WT and Mrp1-/- mice (Figure 3A). 2-AAF co-treatment did not further increase the AST levels in either group. A similar trend was observed for ALT (Figure 3B). Thus, the absence of body weight gain in the 2-AAF/pHx-treated Mrp1-/- mice is not associated with increased liver damage at day 3.

The absence of Mrp1 does not reduce liver regrowth after pHx

To establish whether Mrp1 is directly involved in HPC-mediated recovery of liver mass, we determined the liver weight:body weight ratios of all 8 groups (+/- Mrp1;

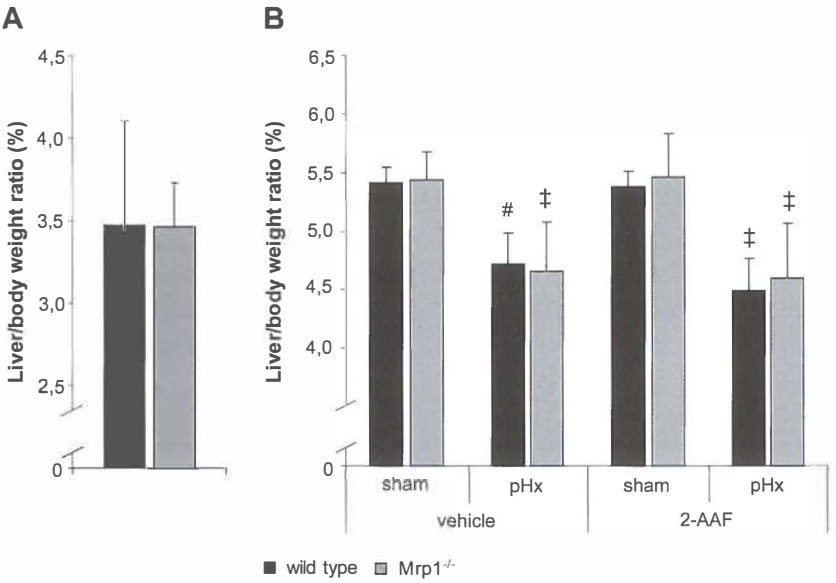

Figure 4. *Liver weight recovery after partial hepatectomy. Mice were weighed directly before surgery. The removed liver mass was weighed directly after removal. The liver to body weight ratio was calculated to determine the relative amount of liver removed during surgery (A). This procedure was repeated at the time of sacrifice to determine the normal liver to weight ratio in sham operated animals and the liver to weight ratio after 3 days of liver regeneration (B). # p < 0,05 versus sham treated animals, ‡ p < 0.01 vs. sham treated animals. Significance was determined by Mann-Whitney test.*

+/- 2-AAF; +/- 70% pHx) of mice at day 3 after surgery (Figure 4). As a result of 70% pHx, both wild type and Mrp1-/- mice lost 3.5% of the total body weight (Figure 4A). At day 3 after surgery, vehicle/sham- and 2-AAF/sham-treated wild-type mice had similar liver to bodyweight ratios (5.42 +/- 0.13 and 5.38 +/- 0.12, respectively) (Figure 4B). Comparable liver to bodyweight ratios were detected for the sham-operated Mrp1-/- mice (vehicle/sham: 5.44 +/- 0.24 and 2-AAF/sham: 5.47 +/- 0.37). Animals that had undergone 70% pHx had significantly reduced liver tissue at day 3 post-pHx compared to sham-treated animals. However, no difference was observed between pHx-treated wild type and Mrp1-/- mice with or without 2-AAF cotreatment (WT: vehicle/pHx 4.72 +/- 0.26 and 2-AAF/pHx 4.49 +/- 0.28; Mrp1-/-: vehicle/pHx 4.66 +/- 0.42 and 2-AAF/pHx 4.60 +/- 0.46). In all 4 pHx-treated groups, the liver weight at day 3 after surgery was restored to approximately 85% of the original liver weight (WT: vehicle/pHx 87.2 +/- 4.7% and 2-AAF/pHx 83.4 +/- 5.2%; Mrp1-/-: vehicle/pHx 85.7 +/- 7.7% and 2-AAF/pHx 84.2 +/- 8.4%).

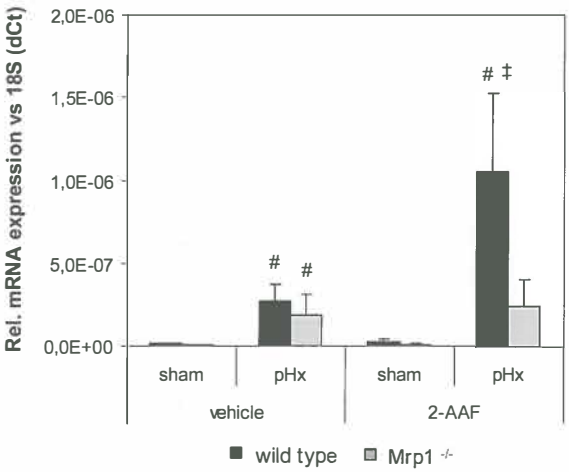

Figure 5. *Hepatic progenitor cell activation is negatively affected in Mrp1-knockout mice. To analyze progenitor cell activation the alpha fetoprotein (AFP) mRNA expression was determined by Q-RT PCR. Expression was normalized to 18S expression and represented as relative mRNA expression (2^-ΔCt). Data show means + sd (n=5). # p < 0,05 vs. sham treated group. ‡ p < 0,05 vs. vehicle/pHx treated group. Statistics determined by Mann-Whitney test.*

Mrp1 deficiency blocks alpha-fetoprotein induction in pHx/2-AAF-treated mice.

Messenger RNA levels of alpha fetoprotein (AFP) were determined as a marker for stem cell activation/differentiation in the livers of all 8 treatment groups (Figure 5) (19). As expected, AFP expression was low in sham operated wild-type and Mrp1-/- mice and 2-AAF treatment did not affect its expression. AFP expression was significantly increased in livers at day 3 in PHx-treated animals and to a similar level in wild type and Mrp1-/- mice. Combined treatment of 2-AAF and pHx resulted in a strong 3.8-fold increase in AFP expression in wild type mice compared to pHx alone. In contrast, 2-AAF treatment did not increase AFP expression in pHx-treated Mrp1-/- mice, indicating a lack of HPC activation in these mice.

Increased numbers of Ki-67 positive cells in wild type and Mrp1-/- livers 3 days after 2-AAF/pHx-treatment

Surprisingly, neither 2-AAF nor the absence of Mrp1 resulted in decreased liver regrowth at day 3 after pHx, even though AFP expression was clearly affected. To determine whether the number of proliferating cells at that time point is different in one or more of the 8 treatment groups, Ki-67 immunohistochemistry was performed on liver sections. The Ki-67 antigen is present in all stages of the cell cycle except for the G0 phase. As expected, no Ki-67 positive cells were detected in livers from sham operated wild type and Mrp1-/- mice, with or without 2-AAF treatment (Figure

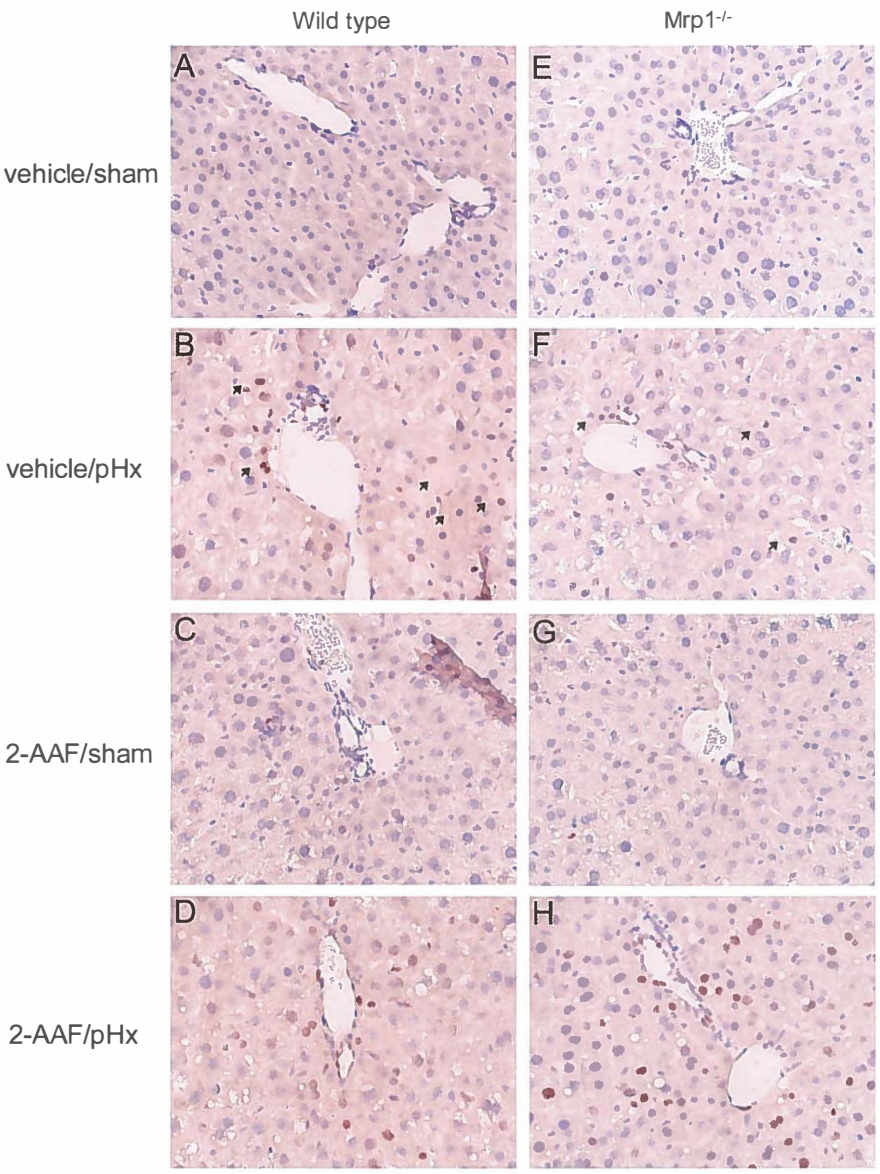

Figure 6. *Proliferation is increased after combined 2-AAF treatment and partial hepatectomy. To determine which cells were proliferating in regenerating liver, Ki-67 staining was performed liver slices from partial hepatectomy (A,C,E,G) or sham (B,D,F,H) operated animals. Staining was performed on liver slices obtained from both wild type animals (A-D) as well as Mrp1-/- (E-H) animals treated with vehicle (A-B, E-F) or 2-AAF (C-D,G-H). Representative pictures from portal areas are shown (original 20x magnification).*

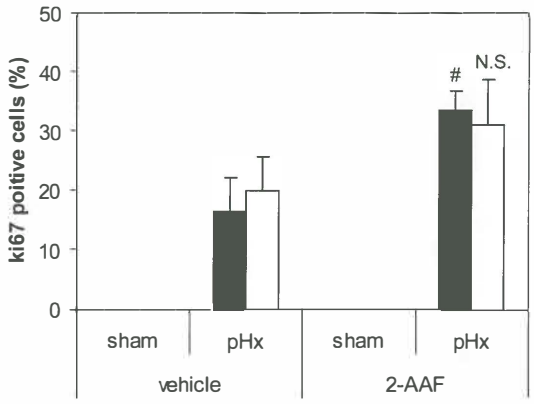

Figure 7. *Proliferation is not affected by Mrp1 knockout. To determine the number of proliferating cells in regenerating liver, the Ki-67 staining were quantified. Ki-67--positive and -negative cells were counted in 3 representative fields per stained slide, correlating with +/- 570 counted cells per slide. # p < 0,001; N.S. not significant. Significance was determined by Mann-Whitney test (n=5 per condition).*

6A, C, E, G). In contrast, Ki-67 positive cells were readily detectable in vehicle/pHx-treated mice, both wild type and Mrp1-/-, indicating that the regenerative process was ongoing. Ki-67 positive cells were detected throughout the liver parenchyma (Figure 6B, F). The number of Ki-67 positive cells was more pronounced in livers from 2-AAF/pHx wild type and Mrp1-/- mice (Figure 6D, H). Ki-67 positive cells were detected throughout the liver but appeared slightly concentrated in the pericentral and periportal regions. The number of Ki-67 positive cells were quantified in representative fields (Figure 7). The percentage of Ki-67 positive cells was comparable in pHx-treated wild type and Mrp1-/-mice (16.6 +/- 5.7% and 20.1 +/- 5.5%, respectively). 2-AAF treatment strongly increased the number of Ki-67 cells in pHx-treated wild type and Mrp1-/- mice, but no significant difference was detected between these groups (wild-type 33.5 +/- 3.3% and Mrp1-/- 31.1 +/- 7.6%). Immunohistochemical staining for active caspase-3 was negative in all groups, to exclude the possibility that the 2-AAF-induced proliferation was due to increased apoptotic cell death (data not shown).

MRP expression during liver regeneration.

Basal Mrp3 expression is increased in the livers of Mrp1-/- mice (Figure 1). As Mrp1 and Mrp3 transport similar substrates, they may compensate for each other. Therefore, we next determined whether pHx, either alone or in combination with 2-AAF treatment, affects hepatic expression of Mrp's and Mdr's in wild type and Mrp1-/- mice, as described before in rats (10). Quantitative real-time PCR was performed on liver samples taken three days after pHx (Figure 8). In wild type mice, Mdr1a and

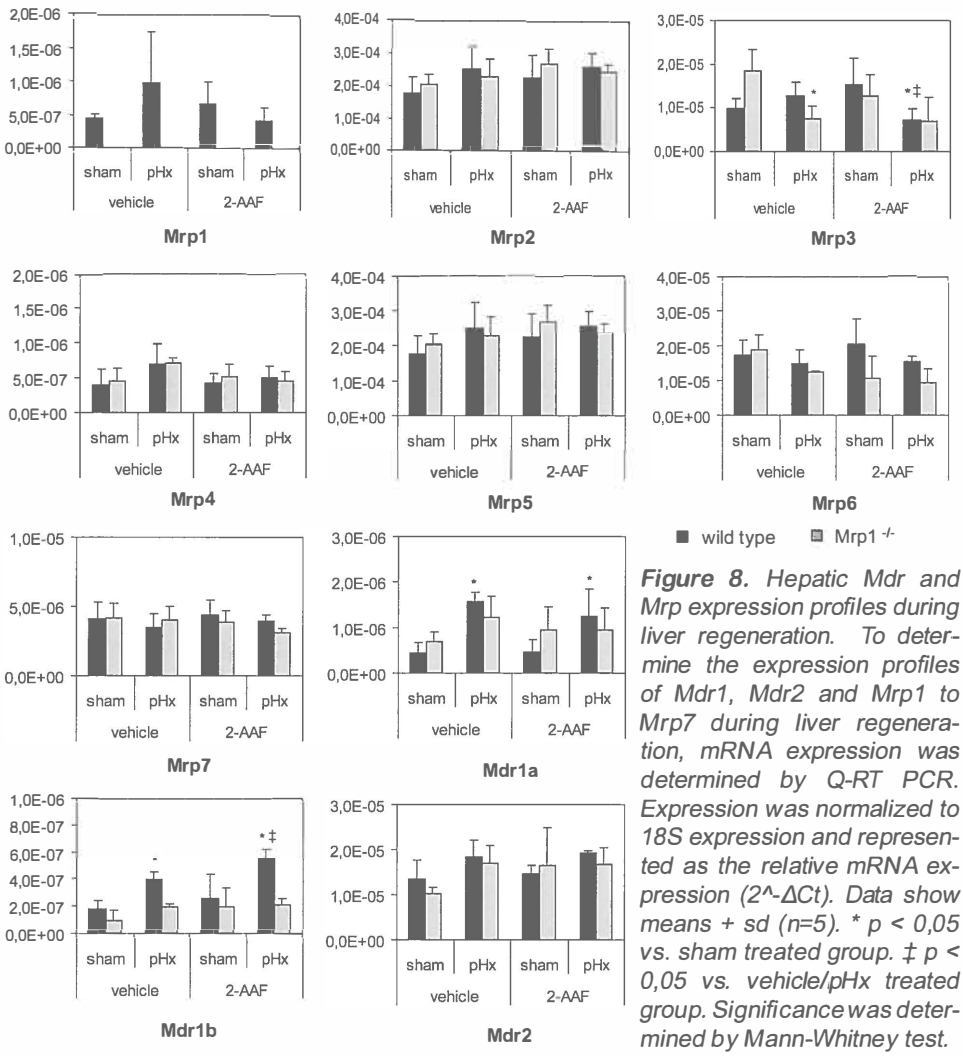

Figure 8. *Hepatic Mdr and Mrp expression profiles during liver regeneration. To determine the expression profiles of Mdr1, Mdr2 and Mrp1 to Mrp7 during liver regeneration, mRNA expression was determined by Q-RT PCR. Expression was normalized to 18S expression and represented as the relative mRNA expression (2^-ΔCt). Data show means + sd (n=5). * p < 0,05 vs. sham treated group. ‡ p < 0,05 vs. vehicle/pHx treated group. Significance was determined by Mann-Whitney test.*

Mdr1b expression is increased after pHx compared to sham-treated animals, both in the vehicle- as well as in the 2-AAF-treated group. In addition, Mdr1b expression was significantly increased after combined 2-AAF/pHx-treatment compared to pHx-treated animals. Surprisingly and in contrast to rats, Mrp3 expression was decreased in 2-AAF/pHx-treated wild-type mice compared to sham-operated and pHx-treated wild type mice. Moreover, expression of Mrp1 was relatively low in all treatment groups and no significant changes were observed after 2-AAF, pHx or 2-AAF/pHx-treatment.

In Mrp1-/- mice, expression of all investigated genes remained the same in the 4 treatment groups, except for Mrp3. pHx reduced Mrp3 expression compared to sham operated animals and 2-AAF treatment did not affect Mrp3 expression.

When wild type and Mrp1-/- mice are compared, differential regulation of Mdr1a, Mdr1b and Mrp3 is observed. Mdr1a and Mdr1b expression is induced in pHx-treated wild type mice, but not in Mrp1-/- mice. Mrp3 expression remains unchanged in pHx-treated wild type mice, but is reduced in pHx-treated Mrp1-/- mice. In contrast, Mrp3 is reduced in 2-AAF/pHx-treated wild type mice and is unchanged 2-AAF/PHx-treated Mrp1-/- mice.

Discussion

In this study, we show that in mice Mrp1 is required for the induction of alpha-fetoprotein during liver regeneration when hepatocyte proliferation is blocked by 2-AAF. This suggests that Mrp1 is required for activation of stem cells to regenerate liver mass. However, 3 days after pHx, liver mass recovery was similar in pHx/2-AAF-treated wild type and Mrp1-/- mice. This implies the existence of hepatocyte- and alpha-fetoprotein-independent mechanisms that contribute to liver regeneration in mice.

MRP1 was previously shown to be highly expressed in the hepatic stem cell compartment in human liver disease (9), which is morphologically detected as a ductular reaction. Similarly, high levels of Mrp1 were observed in the hepatic stem cell compartment in hepatectomized rats exposed to 2-AAF (10), which blocks hepatocyte proliferation. Following our observation that MRP1 expression is also increased in the regenerative compartment of the inflamed intestine and that it provides protection against cytokine-induced cell death (16), we aimed to determine here its role in stem cell-dependent liver regeneration.

First, we compared the hepatic Mrp/Mdr expression profile in wild type and Mrp1-/- mice to assess possible compensatory regulatory mechanisms due to the absence of Mrp1. The “basal” Mrp/Mdr-expression profile was similar in wild-type and Mrp1-/- mice. Significant differences were only observed for Mrp3 and Mdr1b. Absence of Mrp1 leads to an increased expression of Mrp3. Structurally, Mrp3 is the closest homolog of Mrp1, which is reflected in an overlapping substrate specificity (e.g. LTC4, glutathione) (20). It is therefore likely that increased Mrp3 expression compensates for the absence of Mrp1 under normal conditions. The expression of hepatic Mdr1b is significantly lower in Mrp1-/- mice compared to wild-type animals. However, the

levels of Mdr1b in wild type murine liver are already very low. Therefore, the relevance of the decrease in Mdr1b expression is questionable, although it could represent effects in a low-abundant cell type in the liver. This needs further investigation.

Next, we exposed wild type mice to 70% hepatectomy in the absence and presence of 2-AAF. On average, the remaining liver mass was reduced to 35% during partial hepatectomy. 3 Days after surgery, the livers had regrown to approximately 85% of normal liver weight (e.g. sham-operated animals), which correlates well with previous studies (2,2) Notably, regain of liver mass was similar in the presence of 2-AAF. 2-AAF is metabolized by hepatocytes and sulphate-conjugated metabolites block hepatocyte proliferation at the G1/S phase transition (21-23). Liver regeneration then depends on activation and differentiation of stem cells. However, strong ductular reactions were not detected in pHx/2-AAF treated mice in our study. This is in line with recent data reported by Jelnes et al (24). A side-by-side comparison of the pHx/2-AAF model in rats and mice revealed a lack of ductular reactions in mice, whereas it was strongly induced in rats. Still, it has been shown by others that 2-AAF inhibits hepatocyte proliferation in hepatectomized mice also (5). Approximately 70% of the hepatocytes were Ki-67 positive 2 days after pHx, while only 15% were Ki-67 positive in the pHx/2-AAF livers. Three days after pHx, we detected the opposite balance of Ki-67 positive cells comparing pHx- (17% Ki-67 positive cells) and pHx/2-AAF-(34% Ki-67 positive cells) treated wild type mice. Peak hepatocyte proliferation is normally detected 1-2 days after pHx (25,26) and this may explain the relatively low number of Ki-67 positive cells 3 days post pHx. The increased number of Ki-67 positive cells in pHx/2-AAF treated mice may arise from stem cells (from hepatic or extrahepatic origin) that start to proliferate in response to the block of hepatocyte proliferation. However, the interpretation of the Ki-67 staining may also be troubled by the fact that 2-AAF causes a block of cell division in the G1/S phase. Such cells may also appear as Ki-67 positive cells. Still, 3 days after pHx, the number of Ki-67 positive cells is significantly higher in 2-AAF treated mice, either indicating a delayed peak of liver regeneration or an effective block of hepatocyte proliferation. Irrespective of the cellular effect, liver mass recovery at day 3 was similar in pHx and pHx/2-AAF treated wild type mice.

The observation that AFP expression is strongly increased in pHx/2-AAF treated mice suggests the participation of stem cells in the regeneration process. Jelnes et al. suggested that also proliferating mouse hepatocytes may express low levels of AFP (24). Indeed, we observed increased AFP expression in regenerating livers in the absence of 2-AAF. However, the AFP was significantly super-induced (4-

fold) in pHx/2-AAF treated mice, strongly suggesting the involvement of stem cells. Remarkably, the 2-AAF-mediated super-induction was completely absent in Mrp1-/- mice. Thus, Mrp1 is required for induced AFP expression in the liver when hepatocyte proliferation is blocked. The question remains, what is the origin of the cells that are responsible for liver regeneration in pHx-2-AAF treated Mrp1-/- mice. Ductular reactions are not evident in Mrp1-/- or wild type mice. Stem cells may be recruited from the bone marrow to the liver to aid to liver regeneration. Bone marrow cells have been shown to obtain a hepatic gene expression profile in pHx/2-AAF treated mice (5) and Mrp1 activity has been detected in mouse bone marrow stem cells (15). However, AFP was clearly induced in these bone marrow cells (5), which was not detected in the pHx/2-AAF treated Mrp1-/- mice in our study. As the liver regrowth is not significantly reduced in these mice, the AFP-negative cells responsible for this phenomenon remain to be identified. The pHx/2-AAF-treated Mrp1/- mice recover badly from pHx, as shown by the lack of body weight gain between day 2 and 3. Still expression of typical hepatocyte markers genes (Mrp2, Mdr2, HNF4a-not shown) was similar in all 8 animal/treatment groups, indicating the presence of similar numbers of functional hepatocytes at day 3 after pHx. It is remarkable, though, that no induction of Mrp1 mRNA was observed in pHx/2-AAF treated wild type mice. This is in contrast to earlier observations of increased levels of Mrp1 and Mrp3 in stem cell-based liver regeneration in rats and humans (9,10). For Mrp3, we even detected lower expression levels in pHx/2-AAF-treated wild type mice compared to pHx-treated animal. This suggests that regulation of Mrp expression during liver regeneration is species specific and/or time dependent.

Taken together, this study suggests that there are several redundant mechanisms involved in the regeneration of liver mass, including one in mice that is independent of hepatocyte proliferation and induction of the stem cell marker alpha fetoprotein.

Footnotes

The authors would like to thank Andre Zandvoort, Michel Weij and Annemiek Smit-Van Oosten for performing the partial hepatectomy.

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CHAPTER 5

MRP1 expression in human intestinal and hepatic epithelial cells is not suppressed by PPARα

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Abstract

The multidrug resistance-associated protein 1 (MRP1) is an important drug target to treat cancer, but also for inflammatory bowel disease and liver fibrosis. Therefore, it is important to understand the mechanisms that regulate MRP1 expression to explore possibilities for MRP1-directed therapy. Until now, very little is known about the transcriptional regulation of human MRP1. Recent data indicate that intestinal expression of mouse Mrp1 is suppressed by the peroxisome proliferator-activated receptor alpha (PPARα). PPARα acts in concert with the Retinoid X receptor-alpha (RXRα) to regulate fatty acid metabolism. The activity of PPARα and RXRα is controlled by selective ligands. Several pharmaceutical compounds activate PPARα and are used in the treatment of hypercholesteremia.

In this study, we aimed to determine whether PPARα regulates human MRP1 expression in intestinal and liver epithelial cells.

The human colon carcinoma cell lines DLD-1 and Caco-2 and the human hepatoma cell line HepG2 were exposed to PPARα (WY14,643 and GW7647) and/or RXRα agonists (9-cis retinoic acid; 9cRA), in the absence and presence of transiently-transfected PPARα /RXRα. MRP1 expression was determined by quantitative PCR, western blot analysis and immunofluorescence microscopy.

Significant expression of PPARα and MRP1 is detected in DLD-1, Caco-2 and HepG2 cells. WY14,643 and GW7647 induced expression of well-established PPARα target genes (CPT1A and HMG-CoA), but did not change MRP1 mRNA levels. MRP1 expression was not affected by PPARα overexpression, 9cRA treatment significantly reduced MRP1 expression in HepG2 (-77%) and DLD-1 (-57%) cells. The 9cRA-mediated suppression of MRP1 was attenuated by WY14,643 and GW7647.

Our results show that human MRP1 is not regulated by PPARα. In contrast, MRP1 expression is inhibited by the RXRα ligand 9-cis retinoic acid.

Introduction

The multidrug resistance-associated protein 1 (MRP1/ABCC1) is a 190 kDa membrane-bound protein. It is a member of the ATP-binding cassette (ABC) transporter superfamily and exports a wide range of endo- and exogenous compounds out of cells .

MRP1 was discovered in 1992, when its corresponding gene (now called ABCC1) was cloned and shown to confer resistance to the lung cancer cell line H69AR against doxorubicin (1). Further characterization of MRP1 revealed that it belongs to the same family of proteins as the multidrug resistance protein 1 (MDR1/ABCB1/Pgp), a protein that is absent in highly drug resistant H69AR cells. MRP1 is a genuine multidrug transporter as it has been shown to confer resistance to a wide variety of anti-cancer drugs, including Vinca-alkaloids (2), anthracyclines, etoposide (3,4), platinum based drugs (2) and epipodophyllotoxines (reviewed in 2,6)

In addition to its function in drug resistance, substantial evidence demonstrate an important physiological function of MRP1 as well. MRP1 transports endogenous compounds like the pro-inflammatory molecule leukotriene C4 (5), glutathione-, sulphate- (6) and glucuronide- (7) conjugates and (oxidized) glutathione (GSSG + GSH). It is present in specific cell types throughout the body. MRP1 is present in epithelial cells of the blood brain barrier, kidney and testis. Furthermore, relatively high MRP1 levels are found in (activated) murine and human T-helper cells (8,9), in organ-specific stem cells in the intestine (10) and liver (11,12) and in activated hepatic stellate cells that cause liver fibrosis (13). Recent data suggest an important, but paradoxal function for MRP1 in controlling cell survival during inflammatory conditions. MRP1 was shown to sensitize T lymphocytes for anti-Fas-induced apoptosis (14-16), while it protects intestinal epithelial cells under these conditions (10). The dichotomal effect may be a direct effect of cell type-specific production of compounds that are transported by MRP1. The regulation of MRP1 transcription and/or function is therefore of significant therapeutic value as it may dampen an overactive immune system and at the same time prevent excessive organ damage.

Unfortunately, very little information is available about the transcriptional and post-translational regulatory mechanism that control MRP1. Most data are obtained by analyzing the regulation of MRP1 by anti-cancer drugs in cancer cell lines (17-20). In line with this, significant induction of MRP1 expression was observed in vivo in bladder cancer cells after i.v. treatment with doxorubicin (21). In addition, high MRP1 expression was found in human glioma compared to normal brain tissue (22).

In an attempt to identify the regulatory mechanism of MRP1 expression, the human and rodent MRP1 promoter have been cloned. Conserved regions were detected and several potential binding sites for transcription factors have been identified, including those for activator protein 1 (AP-1) and Sp-1 (23). However, the c-jun/fos complex that binds to the AP-1 site and is involved in several signal transduction routes related to inflammation and cell cycle, appears not to be involved in the regulation of MRP1 transcription (24). p53, a key protein in the development of tumor cells, was shown to inhibit expression of MRP1, but the molecular mechanism remains unclear (25). Wang&Beck and Sullivan et al found that binding of transcription factors to the Sp-1 site in the MRP1 promoter was blocked by p53 (25,26). In contrast, Muredda et al. showed that the p53-dependent inhibition of MRP1 is independent of Sp-1 sites (23).

Of considerable pharmacological interest is the observation by Hirai et al. who detected reduced Mrp1 mRNA levels in the intestine of mice treated with synthetic agonists for the peroxisome proliferator-activated receptor alpha (PPARα) (27). PPARα is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily. PPARα is expressed predominantly in tissues that have a high level of fatty acid catabolism, such as liver, heart, and muscle, but lower levels are also detected in the intestine PPARα regulates the expression of a number of genes critical for lipid and lipoprotein metabolism and PPARα agonists are used for the treatment of dyslipidemia. Remarkably, the PPARα-mediated suppression of Mrp1 appeared organ-specific as hepatic Mrp1 expression was not affected in mice treated with PPARα agonists (28).

To evaluate the potential of PPARα as a therapeutic target to manipulate MRP1 levels in human disease, we analyzed the putative involvement of PPARα in the regulation of human MRP1 in intestinal and hepatic cell lines.

Materials and methods

Cell culture

The human hepatoma cell line HepG2 was maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (fungizone, 25 ng/ml) (psf).

The colon carcinoma cell line DLD-1 (DZSM, Braunschweig, Germany) was maintained in RPMI-1640 medium with Glutamax, supplemented with 10% FBS (Invitrogen, Breda, The Netherlands) and psf. Cells were passaged twice a week.

The colon carcinoma cell line Caco-2 was maintained in DMEM (Invitrogen, Breda, The Netherlands) supplemented with 10% FBS, non-essential amino acids (Invitrogen, Breda, The Netherlands) and psf (Biowhittaker, Verviers, Belgium). Cells were passaged twice a week.

For PPARα stimulation, cells were incubated with indicated concentrations of synthetic PPARα agonists GW7647 (Sigma, Zwijndrecht, The Netherlands) or WY14,643 (Sigma) or DMSO (Sigma) as a control. For RXRα stimulation, cells were incubated with 9-cis retinoic acid (Sigma).

PPARα and RXRα transfections

DLD-1 cells were seeded in 6-well plates at 300.000 cells per well. The cells were transfected with plasmid pSG5-PPARα, which was kindly provided by the laboratory of pediatrics (University Medical Center Groningen, Groningen, The Netherlands). Transfection was performed using the Bio-Rad TransfectinTM Lipid reagent protocol (Bio-Rad laboratories B.V., Veenendaal, The Netherlands) according to the manufacturer's instructions. A Transfectin to DNA ratio of 3:1 was used.

HepG2 cells were seeded at 400.000 cells per well. Cells were grown to 70-80% confluency before transfection. HepG2 cells were transfected using the calcium phosphate coprecipitation method (29). Transfections were performed using 100 ng of pSG5-PPARα 100 ng of pSG5-h RXRα (30) and 800 ng pCMV, or with 10 µg of pSG5-PPARα.

Isolation and quantitative RT-PCR

Total RNA was isolated using the TRIzol isolation method according to the manufacturers instructions (Invitrogen Life technologies, Breda, Netherlands) Reverse transcription was performed in a final volume of 50 µl. Messenger RNA (mRNA) levels of 18S, PPARα, MRP1, CPT1A and HMG-CoA-synthase were quantified using the ABI PRISM 7700 (Applied Biosystems, California, USA). Real-time PCR conditions were as described previously (12). For quantification of mRNA levels, the Ct-value difference was calculated between the gene of interest and the housekeeping gene 18S. This delta Ct was corrected to a linear scale (2-ΔCt) to obtain a relative amount of mRNA compared to 18S.

Protein isolation and western blot analysis

Total cell lysates were prepared. Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as a standard.

For western blot analysis of MRP1 protein expression, 15 µg of protein was loaded and separated on 7,5% SDS-PAA gels. Proteins were transferred to a nitrocellulose membrane using tank blotting. Membranes were blocked using 2% milk and 0,5% BSA in PBS/Tween (0,05%). MRP1 was detected using the rat monoclonal antibody Mrpr1 (1:500, Signet, TEBU-Bio) followed by horseradish peroxidase-labeled rabbit anti-rat antibody (1:2000, DAKO). Development of the blot was performed using Supersignal West dura extended Supersignal kit (Perbio Science, Etten Leur, the Netherlands) and signal detection was documented using a Biorad Photodoc system (Biorad, Veenendaal, The Netherlands). Finally, Quantity One software (Biorad, Veenendaal, the Netherlands) was used for to quantify protein levels.

Luciferase assay

The luciferase construct with a PPRE site in its promoter region was a generous gift of Nicolette Huijkman (Dept. of Pediatrics, UMCG, the Netherlands). The luciferase system was provided by Promega. Luciferase activity was determined according to the manufacturers protocol (Promega). Measurements were performed using a microplate luminometer (Berthold detection systems).

Immunofluorescence microscopy

Cells grown on coverslips were washed three times with PBS and fixed with 4% paraformaldehyde (Sigma) for 20 minutes. Permeabilization was achieved using 1% Triton X-100 (Sigma) for 5 minutes. Cells were then incubated with the primary antibody (Mrpr1 (Signet) or PPARα (Santa cruz)) overnight at 4°C. Directly RPE- or Alexa 488-labeled secondary antibodies were incubated for 1 hour at room temperature. Cells were then mounted on fluorescence mounting medium (Dako cytomation, Heverlee, Belgium) analyzed by confocal microscopy (Leica TCS SP2/ AOBS, Rijswijk, Netherland).

Statistical analysis

Experiments were performed in triplicate and analyzed by Student's t-test All statistical tests were performed in SPSS v14.0 for Windows. Significance was defined as $p<0,05$.

Results

Expression of MRP1, PPARα and PPARα target genes in human intestinal and hepatic epithelial cell lines.

First, the endogenous expression levels of PPARα, MRP1 and known PPARα target genes were analyzed in various human intestinal en hepatic cell lines. RNA was isolated from the human colon carcinoma cell line DLD-1, the human colon adenoma cell line Caco-2 and the human hepatoma cell line HepG2. Using quantitative real-time

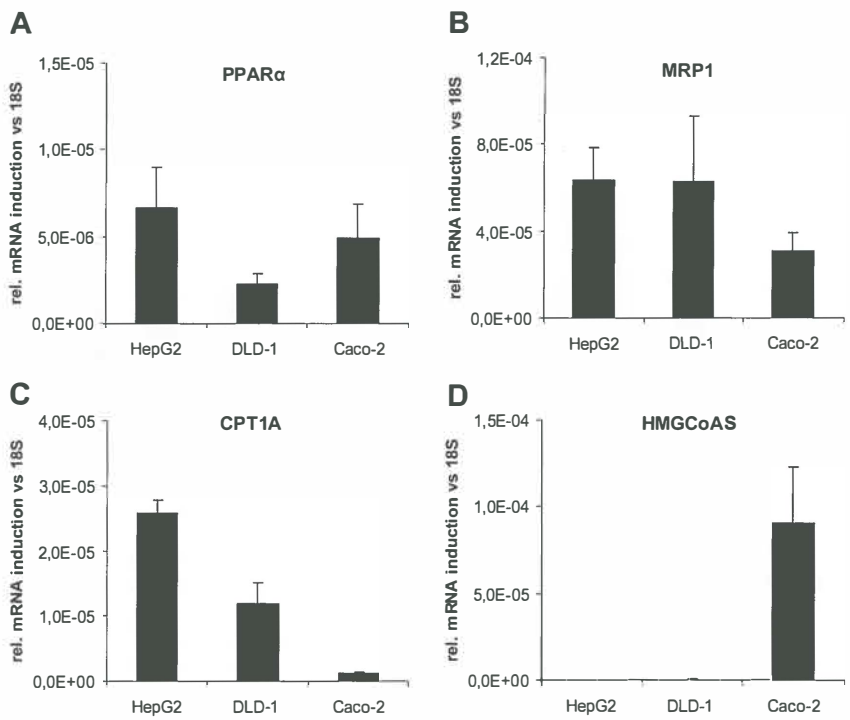

Figure 1. *Expression of PPARα, PPARα target genes and MRP1 in colon and hepatic epithelial cell lines. RNA was isolated from HepG2, DLD-1 and Caco-2 cells. Relative mRNA expression of PPARα (A), MRP1 (B), CPT1A (C), HMGCoAS (D) were determined by Q-RTPCR. Expression was corrected for 18S. Results are represented as means +/- SD.*

PCR, PPARα, MRP1, Carnitin Palmitoyl Transferase I A (CPT1A) and mitochondrial Hydroxymethyl Glucoronide CoA–synthase (HMGCoAS) mRNA expression levels were determined (Figure 1).

PPARα mRNA was present at detectable levels in all cell lines. HepG2 and Caco-2 express similar levels of PPARα mRNA, with approximately 2-fold lower levels in DLD-1 cells. MRP1 mRNA was also readily detectable in all three cell lines. Expression was comparable in HepG2 and DLD-1 cells. Caco-2 cells express approximately 2-fold lower levels of MRP1 compared to HepG2 and DLD-1 cells. Expression of the PPARα target gene CPT1A is highest in HepG2 cells. DLD-1 cells express 2-fold lower, but still significant levels of CPT1A. CPT1A levels in are much lower in Caco-2 cells (> 10-fold) compared to HepG2 and DLD-1 cells. In contrast, HMGCoAS expression was high in Caco-2 cells and undetectable in HepG2 and DLD-1 cells. In following experiments, CPT1A was used as marker for PPARα activation in DLD-1 and HepG2 cells and HMGCoAS as a marker in Caco-2 cells.

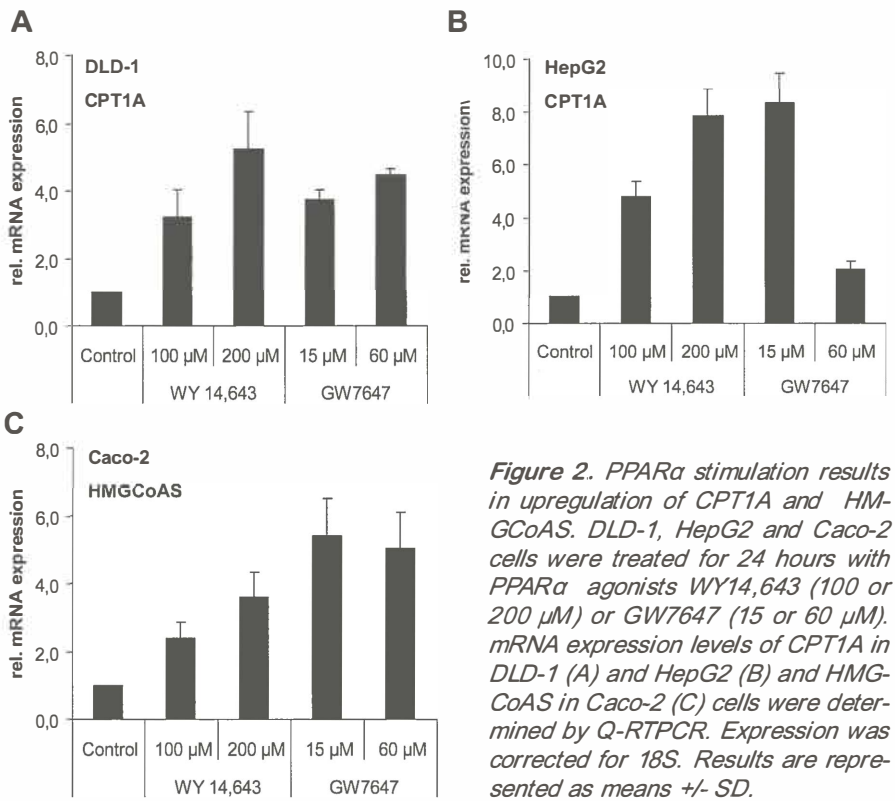

Figure 2. *PPARα stimulation results in upregulation of CPT1A and HMGCoAS. DLD-1, HepG2 and Caco-2 cells were treated for 24 hours with PPARα agonists WY14,643 (100 or 200 µM) or GW7647 (15 or 60 µM). mRNA expression levels of CPT1A in DLD-1 (A) and HepG2 (B) and HMG-CoAS in Caco-2 (C) cells were determined by Q-RTPCR. Expression was corrected for 18S. Results are represented as means +/- SD.*

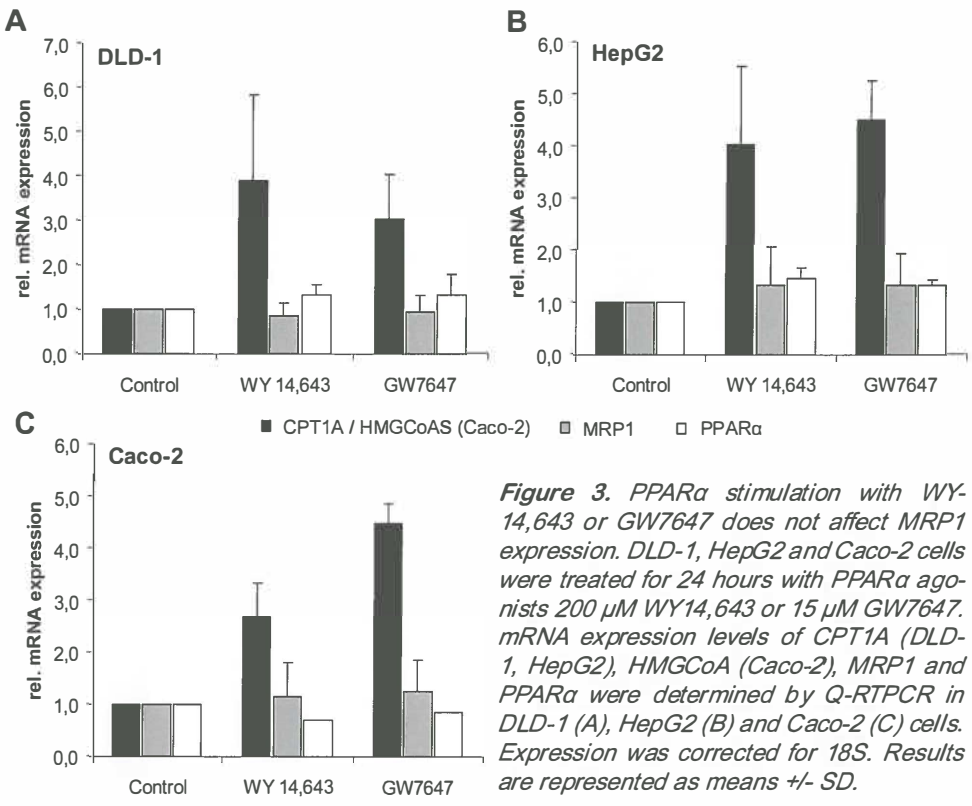

Figure 3. *PPARα stimulation with WY-14,643 or GW7647 does not affect MRP1 expression. DLD-1, HepG2 and Caco-2 cells were treated for 24 hours with PPARα agonists 200 µM WY14,643 or 15 µM GW7647. mRNA expression levels of CPT1A (DLD-1, HepG2), HMGCoA (Caco-2), MRP1 and PPARα were determined by Q-RTPCR in DLD-1 (A), HepG2 (B) and Caco-2 (C) cells. Expression was corrected for 18S. Results are represented as means +/- SD.*

Determination of optimal concentrations of PPARα agonists WY 14,643 and GW7647.

Next, we determined the optimal concentration for analyzing the effect of the PPARα agonists WY14,643 and GW7647 on target gene expression in DLD-1, HepG2 and Caco-2 cells (Figure 2). The induction of PPARα target genes by WY14,643 was dose-dependent and showed strongest effects at 200 uM in all 3 cell lines, leading to 5.3-fold, 7.8-fold and 3.6-fold increases in DLD-1 (CPT1A), HepG2 (CPT1A) and Caco2 (HMGCoAS), respectively. In addition, treatment of all cell lines with 15 µM GW7647 lead to the maximal induction of PPARα target genes. Increasing the concentration of GW7647 to 60 µM did not further increase the expression of the marker genes in DLD-1 and Caco-2 cells. In HepG2 it actually lead to a lower induction of CPT1A. The mRNA levels of the PPARα target genes was not further increased after prolonged (36 h) treatment with PPARα agonists (data not shown). WY14,643 and GW7647 did not change the expression of PPARα itself (data not shown).

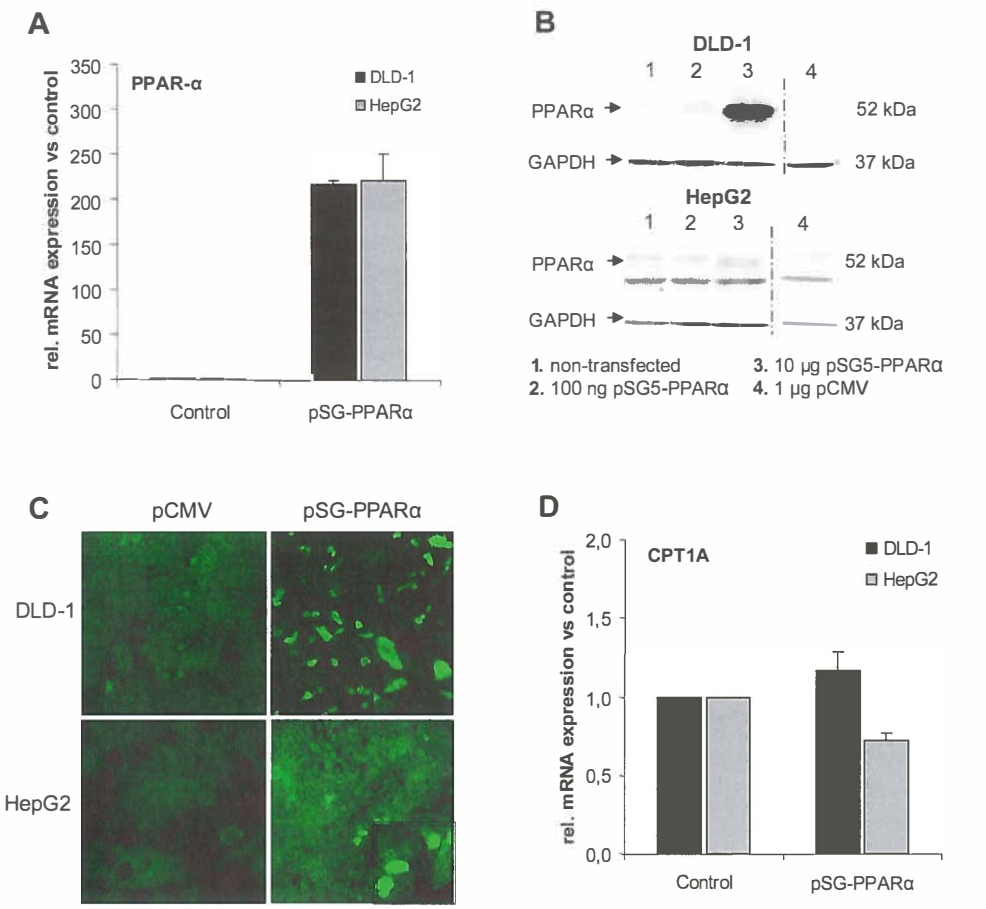

Figure 4. *PPARα overexpression in DLD-1 and HepG2 cells. DLD-1 and HepG2 cells were transfected with pSG5-PPARα leading to a >200-fold increase in PPARα mRNA levels (A). Western blot was performed to analyze PPARα protein expression in untransfected cells and cells transfected with 100 ng, 10 µg pSG5-PPARα or 1 µg pCMV (B). Immunofluorescence microscopy was performed for PPARα to determine transfection efficiency and subcellular location of PPARα after transfection with 10 µg pSG5-PPARα (C). mRNA expression levels of CPT1A (D) and MRP1 (E) were determined in cells transfected with 1 µg pCMV (control) and cells transfected with 10 µg pSG5-PPARα.*

MRP1 mRNA levels are not changed by PPARα stimulation

Next, HepG2, DLD-1 and Caco-2 cells were incubated for 24 h with 200 µM WY14,643 or 15 µM GW7647 and analyzed for transcriptional regulation of MRP1 by Q-PCR (Figure 3). PPARα activation did not result in a change of MRP1 transcript levels (neither induced nor reduced) in any of the human cell lines tested. As established before, CPT1A or HMGCoAS expression was induced in all cases, while PPARα expression itself was not affected by WY14,643 or GW7647 treatment.

Transient PPARα overexpression does not affect PPARα target gene or MRP1 expression

The data presented above suggest that PPARα does not regulate MRP1 expression in human cell lines. However, the absence of a regulatory response towards PPARα ligands may also be due to the presence of limited amounts of PPARα in these cells, which may favour induction (CPT1A, HMGCoAS) rather than repression (MRP1) of specific target genes. To maximize the effects of PPARα stimulation, HepG2 and DLD-1 cells were transiently transfected with expression plasmids for PPARα (pSG5-PPARα) and RXRα (pSG5-h RXRα). PPARα mRNA levels were increased over 200-fold in DLD-1 and HepG2 cells transfected with pSG5-PPARα compared to control (Figure 4A), which was accompanied by a significant overproduction of PPARα protein in both cell types (Figure 4B). The amount of PPARα protein produced appeared significantly higher in DLD-1 cells compared to HepG2 cells. Immunofluorescence microscopy showed that approximately 50% of the DLD-1 cells showed clear PPARα positive nuclei after transfection. In case of HepG2 cells, this was observed for only approximately 10% of the cells (Figure 4C). Endogenous levels of PPARα in both cell lines were hardly detectable by immunofluorescence microscopy (see Figure 4C, pCMV-tranfected cells). Unexpectedly, the increased PPARα levels did not affect the GW7647 induced expression of CPT1A (Figure 4D), nor the mRNA levels of MRP1 (Figure 4E). GW7647 lead to a 3-fold increase of CPT1A in untransfected HepG2 cells (2.7-fold) as well as in PPARα/RXRα- (2.9-fold), or pCMV-control transfected cells (2.8-fold) (data not shown). MRP1 mRNA levels were unchanged in all conditions (+/- transfection; +/- GW7647 treatment). Also when analyzed at the cellular level by immunofluoresence microscopy, expression of MRP1 appeared the same in cells with strong PPARα-positive nuclei and cells without increased PPARα expression (Figure 5, shown for HepG2 cells)

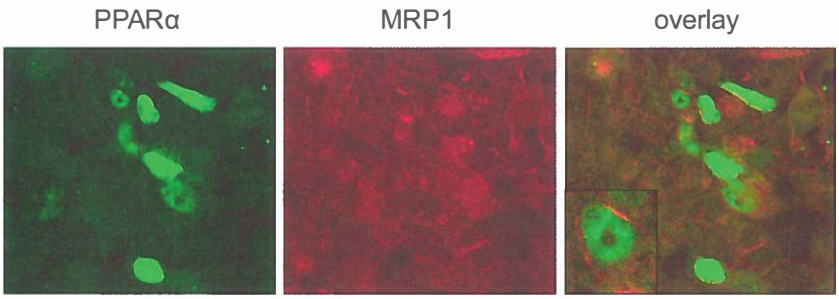

Figure 5. *PPARα overexpression does not affect MRP1 protein expression at the cellular level. HepG2 cells were transfected with 100 ng pSG5-h RXRα and 10 µg pSG5-PPARα. Subsequently, cells were stimulated for 24 hours with 15 µM GW7647. Cells were stained for MRP1 and PPARα and analyzed by confocal microscopy. Clear cellular staining of PPARα was detected in transfected cells (left panel). MRP1 staining located at cellular membranes was readily detected in cells with high and low (/no) PPARα expression (middle panel and overlay in right panel). The inset in the righ panel shows a HepG2 cell with clear nuclear PPARα staining together with MRP1 staining at the cellular membrane. The intensity of the MRP1 staining is, however, comparable to the surrounding non-transfected cells.*

Transfection of pSG5-PPARα results in expression of functional PPARα

To confirm that the artificially produced PPARα is functional, DLD-1 cells were co-transfected with pSG5-PPARα, pSG5-h RXRα and a luciferase reporter construct containing three PPRE's in front of the luciferase gene (pPPRE-luciferase). After an additional 24 h treatment with GW7647, cell were harvested and analyzed for luciferase activity. Transfected DLD-1 cells with only the pPPRE-luciferase construct contained low luciferase activity (400 AFU), which was not induced by GW7647 (Figure 6). Cotransfection with pSG5-h RXRα resulted in a minor increase in luciferase activity which was not responsive to GW7647 (1,000 AFU). In contrast,

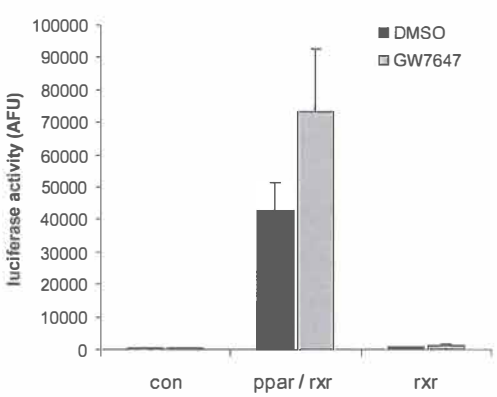

Figure 6. *Heterologously expressed PPARα is active and ligand-inducible. DLD-1 cells were transiently transfected with a luciferase reporter plasmid containing 3 PPARα responsive element (control), combined with 100 ng pSG5-PPARα and 100 ng pSG5-h RXRα (PPARα / RXRα) or only 100 ng pSG5-h RXRα (RXRα). Twenty four (24) h after transfection, cells were treated for an additional 24 h with DMSO (control) or 15 µM GW7647, followed by determination of the luciferase activity in total cell lysates.*

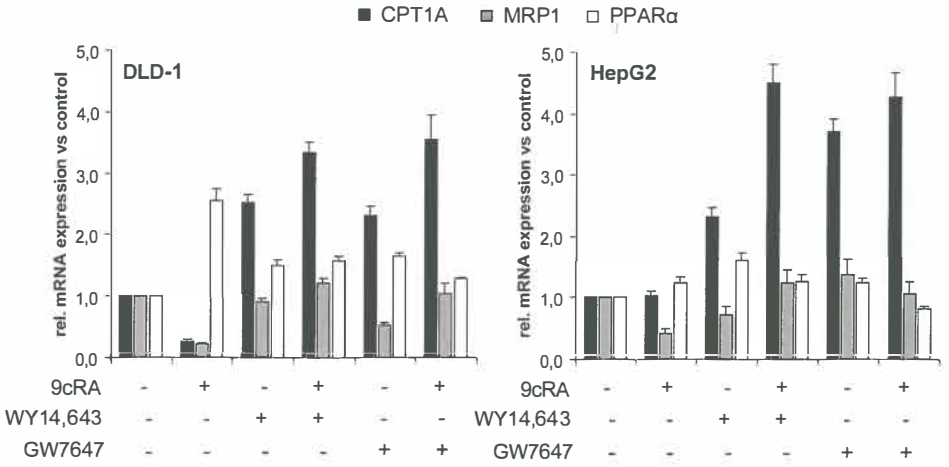

Figure 7. *The RXRα-ligand 9cRA decreases MRP1 expression in DLD-1 and HepG2 cells. DLD-1 cells and HepG2 cells were transiently transfected with 100 ng pSG5-PPARα and 100 ng pSG5-h RXRα. Subsequently, cells were treated for 24 hours with 1 μM 9-cis retinoic acid (9cRA), 200 μM WY14,643, 15 μM GW7647 or a combination. mRNA expression levels of CPT1A, MRP1 and PPARα were determined in DLD-1 (A) and HepG2 (B) cells by Q-RTPCR. Expression was corrected for 18S. Results are represented as means +/- SD.*

co-transfection of DLD-1 cells with pSG5-PPARα, pSG5-h RXRα and pPPRE-luciferase strongly induced luciferase activity ($42*10^3$ AFU). Moreover, GW7647 further increased the luciferase activity 2-fold in these cells ($73*10^3$ AFU). These data show that the artificially produced PPARα is functional.

9-cis retinoic acid downregulates MRP1 in DLD-1 and HepG2 cells and increases PPARα mRNA expression in DLD-1 cells

Finally, we investigated whether RXRα may independently influence the activation of PPARα-regulated gene transcription and/or MRP1. DLD-1 and HepG2 cells were co-transfected with pSG5-PPARα and pSG5-h RXRα to allow maximum activation of PPARα, followed by selective activation by WY14,643, GW7647 and/or the RXRα ligand 9-cis retinoic acid (9c-RA). WY14,643- and GW7647-induced expression of CPT1A was even further increased by 9cRA in both cell types (Figure 7). No change in expression was observed for PPARα and MRP1 in any condition where a PPARα ligand was present. However, when cells were treated with 9cRA alone, a significant reduction in MRP1 mRNA levels was detected in both DLD-1 and HepG2 cells. Remarkably, 9cRA treatment causes a strong induction (2.6-fold) of the PPARα mRNA levels in DLD-1 cells. This was not observed in HepG2 cells.

Discussion

In this study, we show that human MRP1 expression is not suppressed by PPARα in hepatic HepG2 and colonic DLD-1 and Caco-2 cells. Neither PPARα agonist nor PPARα overexpression did affect MRP1 transcription. In contrast, MRP1 mRNA levels were significantly reduced by the RXRα ligand 9-cis retinoic acid. Remarkably, the 9cRA-mediated repression of MRP1 was attenuated by PPARα agonists.

MRP1 is an important drug target. MRP1 levels are increased in many types of cancer and protect malignant cells against cytostatic drugs (2-4). Moreover, increased levels of MRP1 were detected in activated hepatic stellate cells that cause liver fibrosis (13). The other side of the coin is that MRP1 levels are also increased in regenerative tissue in the intestine and liver (10-12) and MRP1 was shown to protect against cytokine–induced cell death (10). Finally, Mrp1 is required for the activation of the adaptive immune response (31). These studies show that there is not one straight approach for MRP1-targeted therapy; for some diseases MRP1 should be inhibited (cancer, liver fibrosis), for others it should be induced (IBD, liver regeneration). Pharmaceutical inhibitors of MRP1 transport activity are known, like MK571 and oleanolic acid, but these compounds are not very selective and are prone to give side effects when used for therapy. Manipulation of the transcriptional regulation of MRP1 would be alternative approach to change MRP1 activity. Unfortunately, to date, little is known about the molecular mechanisms involved in the transcriptional regulation of human MRP1. The recent discovery that PPARα may repress Mrp1 expression in mice opened new possibilities to therapeutically modulate MRP1 expression. PPARα is a ligand-activated transcription factor. Agonists and antagonists are available that allows modulation of Pparα target gene expression depending on the desired therapeutic effect Mrp1 expression is reduced in the intestines of mice that are treated with Pparα agonists (Wy14643 and GW7647) (27). Notably, Mrp1 levels were not changes in the livers of these Wy14643- or GW7647-treated mice. Following these observations, we studied the possible PPARα-mediated regulation of human MRP1 in intestinal and hepatic cell lines. However, we did not detect any significant effect of PPARα agonist on the transcriptional levels of MRP1 in these cell lines. Our experimental conditions showed robust induction of known PPARα target genes by the PPARα agonists Wy14643 and GW7647 in DLD-1, Caco-2 and HepG2 cells. Transient transfection of functional PPARα did not result in changed levels of MRP1 even though transfection efficiencies of 50% were obtained. Also in individual cells with clearly increased levels of PPARα, MRP1 expression appeared

unchanged and was detected in the cellular membrane. These data fail to indicate any role for PPARα in human MRP1 regulation. A possible explanation for these observations is that PPARα is already (partly) activated by endogenous ligands in these cell lines resulting in full repression of MRP1. PPARα agonists may still be able to induce expression of positively regulated target genes, but may not exert an additional effect on suppressed target genes, like MRP1. To explore this possibility, we exposed DLD-1 and HepG2 cells to the PPARα antagonist MK866. Preliminary data reveal that also MK866 does not change MRP1 expression, arguing against a high basal level of PPARα activation in these cells. Taken together, these results do not support a possible role of PPARα in the regulation of human MRP1.

The question remains whether the change in intestinal MRP1 expression in Wy14643- or GW7647-treated mice is a direct effect of PPARα-mediated regulation. The fact that the repression of MRP1 was not observed in Pparα -ko mice seems to support this possibility. However, these results have not been confirmed by a detailed Mrp1 promoter analysis and/or chromatin immune precipitation experiments. Also no putative PPARA response elements (PPREs) have been studied in the Mrp1 promoter element. Therefore, it cannot be ruled out that the observed suppression of Mrp1 in intestines of Wy14643- or GW7647-treated mice is in fact and indirect effect.

The only factor that significantly affected MRP1 transcription in our study was the RXRα ligand, 9-cis retinoic acid (9cRA) leading to a 77% reduction in DLD-1 cells and a 57% reduction in HepG2 cells. Remarkably, this repression was attenuated by PPARα agonists. It remains to be determined whether 9cRA acts through RXRα. It has been reported that high levels of 9cRA may reduce RXRα protein levels (30) thereby reducing the expression of its target genes. However, such a strong reduction in RXRα protein was not detected when HepG2 cells were treated with 1 μM 9cRA (30). Thus, our observations suggest that there is a complex interplay between RXRα and PPARα ligands in relation to MRP1 transcription. In access 9cRA, PPARα agonists induce MRP1 expression. This is opposite to the Pparα-dependent regulation of mouse Mrp1 in the intestine.

In conclusion, our data do not support a role of PPARα in suppressing human MRP1 expression. In contrast, the RXRα ligand was identified as an inhibitor of human MRP1 expression. PPARα agonists attenuate the 9cRA-mediated suppression of MRP1. These data warrant a further analysis of the interplay of PPARα, RXRα and their ligands in the regulation of human MRP1.

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CHAPTER 6

General discussion and summary

A. van Steenpaal

The Multidrug Resistance-associated Protein 1 (MRP1) has been a major subject of scientific and clinical interest ever since its first characterisation in 1992 (1). Its role in the development of multidrug resistance has turned it into an important diagnostic marker and a therapeutic target to increase the efficacy of a variety of drugs, including ones to treat cancer and HIV. However, MRP1 also serves important (patho) physiological functions that are unrelated to drug therapy (reviewed in Chapter 1). These functions are only now being discovered. In this thesis, we studied the (patho) physiological function of MRP1 in intestinal and liver diseases. Specific focus was put on its function and regulation in inflammatory bowel disease (IBD) and liver during regeneration.

The role of MRP1 during inflammation

In chapter 2 of this thesis, we show that the expression of MRP1 is increased in the inflamed intestinal mucosa of IBD patients when compared to the neighbouring uninflamed tissue. High MRP1 expression is observed in two cellular compartments of the inflamed mucosa, the intestinal epithelial cell layer (predominantly in the crypt region) and lamina propria mononuclear cells.

In order to investigate the functions of MRP1 in inflamed intestine, we subjected cells from the human intestinal epithelial cell line DLD-1 and human T-lymphocytes to inflammation factors, e.g. tumor necrosis factor-α (TNF-α) and anti-Fas, in combination with manipulation of the level or activity of MRP1 (Chapter 2 and 3). We found that MRP1 protects intestinal epithelial cells from cytokine-induced apoptosis, while the exact opposite, MRP1 promotes cytokine-induced apoptosis, was observed for T-lymphocytes. This indicates that the effect of MRP1 function differs between cell types, most likely depending on external conditions that affect the cellular status and the production of specific substrates that are transported by MRP1.

The protective effect of MRP1 in DLD-1 cells is related to the MRP1-mediated export of a pro-apoptotic compound from the leukotriene biosynthesis pathway, which is most likely the high affinity substrate of MRP1 leukotriene C4 (LTC4) (Chapter 2). Apoptotic cell death of DLD-1 cells is prevented when the intracellular generation of LTC4 is blocked in combination with cytokine-exposure and MRP1 inhibition. MRP1 blockade has also been shown to prevent angiotensin II-mediated endothelial cell apoptosis thereby confirming a role for MRP1 and intracellular LTC4 generation in modulating cell survival during inflammation (2).

In contrast to its role in epithelial cells, MRP1 expression sensitized T-lymphocytes

for apoptosis (Chapter 3). Two other independent studies have recently shown comparable effects of MRP1 inhibition on T-lymphocytes apoptosis (3,4). The exact mechanism of MRP1-dependent sensitization remains unclear, although we were able to exclude the involvement of the leukotriene biosynthetic pathway. In addition, we have shown that intracellular glutathione is not involved. Therefore, the involvement of MRP1-mediated glutathione export as a cause for T-cell apoptosis remains a subject of discussion (3-5).

Since MRP1 expression increases upon T-lymphocyte activation (6) and is involved in cytokine release (5) a possible physiological function of MRP1 expression in T-lymphocytes could be self-regulation of the immune response. When T-cells are inactive, they express low MRP1 levels, therefore having a low(er) susceptibility to apoptosis and low production of cytokines. During an immune response, MRP1 expression in T-lymphocytes is increased resulting in an increased sensitivity to cytokine-induced apoptosis, active T-lymphocytes are cleared by apoptosis more easily and subsequently the immune response is silenced. Derangements in this process could be important in patients with autoimmune diseases, including inflammatory bowel disease. Modulation of MRP1 levels or activity is therefore an interesting possibility to improve the balance of immune responses in these patients.

The role of MRP1 in tissue regeneration

Several organs are able to regenerate. The intestinal epithelium is continuously renewed by proliferation and differentiation of progenitor cells located at the base of the crypts (reviewed in (7)). During migration of newly formed epithelial cells to the top of the villus, these "naïve" epithelial cells differentiate into fully committed intestinal epithelial cells (enterocytes). At the top of the villus, the "old" enterocytes die from apoptosis and are shed from the colon mucosa. This is a constitutive process that renews the entire epithelial cell layer every 2-5 days in the healthy intestine.

A quite different situation is observed for the liver. Very little tissue regeneration is occurring in the healthy liver, where only 1 in 20.000-40.000 hepatocytes is dividing. Concurrently, the average life span of a hepatocyte is approximately 5 months, around 50-fold longer than an enterocyte. However, the hepatic regeneration machinery is strongly activated after injury that causes loss of functional liver mass (e.g. alcohol, surgical resection or (viral) infection) (reviewed in (8)). Under these conditions, hepatocytes enter cell division and proliferate until the original liver capacity is restored. The process of liver regeneration is initiated by the increased

production of several inflammatory cytokines, e.g. TNF-α and Il-6. In response to the local increase of these cytokines, hepatocytes start to proliferate. During severe liver injury, when hepatocyte proliferation is inhibited (e.g. viral infections, chemical inhibition by drug abuse) the hepatic progenitor cells are activated (8). These cells proliferate and subsequently differentiate into mature hepatocytes or cholangiocytes.

Interestingly, during inflammation in both liver (primary biliary cholestasis, chronic hepatitis C) and intestinal diseases (ulcerative colitis, Crohn's disease), MRP1 expression was observed in the regenerative compartment containing the progenitor cells (9). In addition, an in vivo rat model for severe liver injury revealed MRP1 induction in the progenitor cell compartment during liver regeneration (10). These observations combined suggest a functional role of MRP1 in progenitor cells. Based on the cytoprotective effects of MRP1 in epithelial cells (Chapter 2), we postulated that MRP1 also provides cytoprotection to progenitor cells, thereby ensuring the regenerative capacity of organs under toxic conditions. A common factor in hepatic or intestinal inflammatory disorders and liver regeneration is the increased presence of pro-apoptotic cytokines.

Under normal conditions, MRP1 expression is very low in the liver which corresponds to the quiescent nature of the hepatocytes. In the intestine, where regeneration is continuous, MRP1 is present at much higher levels. In both organs, MRP1 is increased significantly in the progenitor cell compartment during severe inflammation, suggesting a functional role in ensuring tissue regeneration.

To determine whether Mrp1 plays a crucial role in progenitor cell-mediated liver regeneration in vivo, we subjected wild type and Mrp1 knockout mice to partial hepatectomy (pHx) in the presence of the hepatocyte proliferation-blocker, 2-acetylaminofluorene (2-AAF) (Chapter 4). In contrast to our hypothesis, we did not detect a significant reduction in liver tissue recovery in 2-AAF-treated $Mrp1^{-/-}$ and wild type mice 3 days after partial hepatectomy. However, our hypothesis was supported by the observation of a clear induction of the progenitor cell marker α-fetoprotein (AFP) in 2-AAF/pHx-treated wild type, which was absent in 2-AAF/pHx-treated $Mrp1^{-/-}$ mice. The induction of AFP in 2-AAF/pHx-treated wild type mice, however, did not coincide with induction of Mrp1 or any other ABC-transporter tested (Mrp2-7, Mdr1a/b, Mdr2). In contrast, 2-AAF treatment lead to a significant induction of Mrp1, Mrp3 and Mrp4 in partial hepatectomized rats (10). This was analyzed 9 days after pHx, while in our experiments transporter expression in mouse liver was analyzed 3 days after pHx. It remains to be determined whether the discrepancy in Mrp1 expression is species-specific and/or dependent on the exact time point after pHx.

A complicating factor in the analysis of the physiological functions of Mrp's in general is that a significant overlap in substrate specificity exist between the different Mrp's. Mrp3 is the closest structural homologe of Mrp1 with established substrate specificity overlap (11). Of all Mrp/Mdr's tested, actually only Mrp3 mRNA levels appear to be significantly increased in livers of $Mrp1^{-/-}$ mice compared to wild type controls (Chapter 4). This may suggest that compensatory mechanisms are induced in livers of $Mrp1^{-/-}$ to maintain the regenerative capacity under variable conditions. As also Mrp3 was strongly induced in livers of 2-AAF/pHx treated rats, it may be worthwhile to perform the 2-AAF/pHx experiments in $Mrp1^{-/-}/Mrp3^{-/-}$ double knock-out animals to establish whether the combination of these two Mrps is required for optimal progenitor cell-mediated liver regeneration.

Still, the absence of AFP induction in 2-AAF/pHx-treated $Mrp1^{-/-}$ is intriguing and suggest that Mrp1 specifically is required for progenitor activation. Ki-67 positive cells were detected throughout the liver parenchym which does not support a decent of the hepatic progenitor cell niche that is thought to reside in the canals of Hering. This may suggest that also bone marrow stem cells significantly contribute to liver regeneration in 2-AAF/pHx treated mice. Whether AFP levels remain low in the transition of these bone marrow stem cells to hepatocytes needs further investigation.

Regulation of MRP1 expression

It is clear that MRP1 is an important therapeutic target in a variety of diseases, including cancer, HIV, autoimmune diseases -like the IBDs- and liver regeneration. However, inhibiting MRP1 is not the universal therapeutic approach for all these diseases. Especially for IBD and organ regeneration, stimulating MRP1 activity may aid to limit or reduce tissue damage. Early research has been especially focussed on identifying pharmacological inhibitors of the transport activity of MRP1. Most used is MK571, but several additional inhibitors have been identified and characterized in recent years (selective MRP1 inhibitors: (12,13). The problem of these compounds is that they are not selective, even though they are often described as selective inhibitors of MRP1.

An alternative approach to inhibit MRP1 function is to modulate the transcriptional regulation of the corresponding ABCC1 gene. Conditions are known in which MRP1/ ABCC1 expression is changed. For example, prolonged treatment of cancer cell lines with doxorubicin results in development of multidrug resistant clones overexpressing MRP1 (14).

Although many putative regulatory sequences have been identified in the MRP1

promotor, none of them have been unequivocally shown to be involved in the transcriptional regulation of MRP1. One of the best described physiological compounds that regulates MRP1 expression is Il-6. Il-6 treatment has been shown to increase MRP1 expression and activity in human hepatoma cell lines as well as a human keratinocyte cell line (15,16). Il-6 has been shown to reduce MDR expression levels in rat liver (17). Although contradictory effects are observed for the effect of Il-6 on ABC transporter expression, it is clear that Il-6 is involved in the transcription regulation of ABC transporters. It is hypothesized that, during the onset of inflammation, changes in expression levels of hepatic enzymes involved in metabolism are initiated by the release of Il-6.

Recently, an interesting novel transcriptional regulator was identified that may be involved in tissue-specific regulation of Mrp1, namely the peroxisome proliferator-activated receptor alpha (PPAR-α). Mrp1 expression was reduced in the intestines of wild type mice that were treated with synthetic PPAR-α agonists, while Mrp1 levels in the liver were unaffected. The Ppar-α agonist dependent repression of Mrp1 was not detected in *Ppar-α* knock mice. The tissue-selective mechanism involved in Ppar-α-mediated Mrp1 regulation may have value for therapeutic applications where highly tissue-selective manipulation of MRP1 is required. Since no data are available on the putative regulation of human MRP1 by PPAR-α, we studied this in Chapter 5. Unfortunately, PPAR-α did not have any effect on human MRP1 expression in either hepatic or intestinal cell lines. It may be possible that there are many species-specific differences in PPAR-α target gene expression, as already shown by the differences in expression level and location during embryonic development between rodents and humans (reviewed in (18)). However, our results might also indicate that the MRP1-reduction in the intestine does not take place in the intestinal epithelial cell compartment, but in an entirely different cell type. Remarkably, we found that activation of the binding partner of PPAR-α, RXR-α, by 9-cis retinoic acid leads to strong repression of MRP1 expression. In the presence of PPAR-α agonists, this repressive effect was absent. This indicates that there may be a complex interplay between PPAR-α, RXR-α and possibly additional factors that regulates MRP1 expression. It is therefore relevant to further delineate the mechanisms that are involved in RXR-α/9 cis retinoic acid-mediated repression of MRP1. However, as RXR-α is an obligatory partner for many different members of the nuclear receptor family, modulating its activity in vivo is likely to affect many different metabolic processes.

Future perspectives

Until recently, the focus of Mrp1 research was on the "negative" effects of MRP1 expression, e.g. multidrug resistance in cancer tissue. Therefore, the use of MRP1 inhibitors in the treatment of cancer has been the main therapeutic goal. Although this may be beneficial to sensitize multidrug resistant cancer cells to anticancer drugs, our research shows that in inflammatory conditions MRP1 inhibitors may potentially induce epithelial cell damage and T-cell resistance to apoptosis. It has already been demonstrated that adequate MRP1 function is required to prevent methotrexate-mediated intestinal cell damage (19) indicating that enhancing MRP1 function can reduce intestinal toxicity of cytotoxic drugs. Our finding that MRP1 expression sensitized T-lymphocytes to apoptosis is new. The physiological role of MRP1 in T-cells and other immune competent cells such as mast cells, monocytes, macrophages and antigen presenting cells is poorly addressed (20). For instance the Ig- mediated release of cysteinyl leukotriene from mast cells is markedly impaired by MRP1 deficiency, indicating that MRP1 plays an important role in the development of allergic airway inflammation (21). In macrophages, the bacterial cell wall component lipopolysaccharide (LPS) can increase MRP1 expression and function (22). Generally, it is believed that the infection-driven upregulation of MRP1 is necessary to maintain the cellular redox status. As a consequence of increased MRP1 levels these immune competent cells are less susceptible for immunosuppressive drugs. However, in T-cells the opposite seems true, apart from the apparent function of MRP1 in cytokine release (5) its function in the T-cell life cycle needs further exploration. Considering the opposite effect of MRP1 inhibition on apoptosis of epithelial cells and T-cells, it is important to select the right disease and target the right cell for MRP1 inhibition or stimulation experiments. In Crohn's disease, resistance of T-cells to apoptotic cell death is an important negative factor so in this disease T-cells should be targeted for induction of MRP1 expression. In contrast, in ulcerative colitis epithelial cell damage is more prominent and local treatment (for instance via enemas) with enhancers of MRP1 function seems to be the best approach. We showed that MRP1 mediated intracellular LCT_4 accumulation is involved in epithelial cell damage. LCT_4 receptor inhibition has been explored in ulcerative colitis and indeed montelukast, a LCT_4 receptor inhibitor, showed beneficial effects on experimental colitis (23). In addition, it is known that smoking is beneficial for ulcerative colitis. It has already been shown that MRP1 protects airway epithelial cells against damage by cigarette smoking. Interestingly common anti-inflammatory drugs such as budesonide can decrease MRP1 function in pulmonary epithelial cells (24). Therefore, these drugs could be

detrimental for protection of epithelial cells in smoking patients. Since MRP1 function can also be inhibited by dietary components such as the phytochemical glycyrrhetinic acid (found in licorice) the effect of dietary components affecting drug transporters should be explored in IBD and intestinal cytotoxicity (25-28).

In order to predict efficacy and potential side effects it is important to know the effects of MRP1 inhibition and/or overexpression on susceptibility to apoptosis in other diseases and cell types. For instance, Hannivoort et al. have shown that MRP1 protects activated hepatic stellate cells from necrotic cell death (29). Therefore in liver inflammation, MRP1 upregulation could sustain the process of hepatic fibrosis. Secondly, there are many inducers of cell death for which the effect of MRP1 inhibition and/or overexpression has not been investigated yet. Therefore, it is still largely unclear whether MRP1 modulation can be successfully applied without strong side effects due to cytotoxic effects on selected cell types.

Finally, a critical part of the potential use of modulators of MRP1 expression in clinical applications is the investigation of mechanisms that control the transcriptional regulation of human MRP1. Without knowledge of these processes safe manipulation of MRP1 function cannot be applied in either IBD or liver disease.

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CHAPTER 7

Nederlandse samenvatting

A. van Steenpaal

Resistentie tegen geneesmiddelen is een onwenselijke complicatie bij de behandeling van ernstige, vaak chronische ziekten zoals auto-immuunziekten en kanker. Veel onderzoek heeft plaatsgevonden naar de mechanismen die leiden tot geneesmiddelresistentie, waardoor deze voor een gedeelte bekend zijn. Een van de oorzaken van resistentie is de verhoogde aanwezigheid of activiteit van specifieke transporteiwitten. Deze transporteiwitten pompen de geneesmiddelen de cel uit waardoor het zijn werk niet kan doen. Veel van deze transporteiwitten komen ook voor in gezonde/normale weefsels/organen, maar er is nog maar weinig bekend in welke cellen ze precies zitten en wat hun functie daarin is. Het onderzoek, beschreven in dit proefschrift, heeft zich gericht op de natuurlijke functie van één van deze eiwitten in de lever en de darmen. In deze samenvatting wordt een overzicht gegeven van de achtergrond en de, in dit proefschrift beschreven, resultaten.

7.1.1 De darmen

De darmen vormen een belangrijk deel van het verteringssysteem. Hier worden voedingsstoffen uit het voedsel opgenomen en een deel van de, in het lichaam aanwezige, schadelijke stoffen uitgescheiden. Het darmstelsel is opgebouwd uit een aantal verschillende delen. Wanneer voedsel de maag verlaat komt het in de twaalfvingerige darm. Hier wordt het voedsel gemengd met gal dat uit de galblaas de darmen in wordt geperst. De galzouten fungeren als een soort zeep, waardoor vetoplosbare stoffen in het voedsel “oplossen” en opgenomen kunnen worden via de darmwand. Het mengsel van voedsel en gal komt vervolgens in de dunne darm. Hier wordt het voedsel afgebroken door bacteriën en lichaamseigen enzymen en worden essentiële voedingscomponenten opgenomen via de darmwand in het bloed. Via de dunne darm komt de massa in de dikke darm. In dit deel van de darmen worden water en zouten opgenomen uit de darminhoud om vochtverlies van het lichaam tegen te gaan.

De darmwand is een essentieel onderdeel van de darmen. Het is in staat voedingsstoffen op te nemen uit de darminhoud. Daarnaast vormt het de eerste belangrijke barrière die ervoor zorgt dat bacteriën de bloedbaan niet kunnen bereiken. De darmwand bestaat uit verschillende lagen, ieder met een eigen specifieke opbouw en functie. In dit proefschrift gaat het met name om de twee lagen die het dichtst bij de darminhoud zitten, namelijk de mucosa en de lamina propria. De mucosa bestaat uit een laag cellen welke verantwoordelijk is voor uitscheiding van enzymen, betrokken bij het vrijmaken van voedingsstoffen uit de darminhoud, en de daadwerkelijke opname van deze voedingsstoffen en vormt het directe contactoppervlak van de darmen. De oppervlakte cellen worden epitheelcellen

genoemd. De lamina propria is de laag direct onder de mucosa en bestaat voor een groot gedeelte uit bloed- en lymfevaten welke noodzakelijk zijn voor het aanvoeren van voedingsstoffen en een efficiënte immuunreactie.

7.1.2 Inflammatoire darm ziekten

Inflammatoire darm ziekten (IBD; Inflammatory bowel disease) is de verzamelnaam voor chronische ontstekingsziekten van het darmstelsel. IBD kan onderverdeeld worden in de ziekte van Crohn (CD) en colitis ulcerosa (UC). Bij deze aandoeningen ontstaan zweren in de darm, welke lijden tot onder andere pijn, verminderde eetlust en (bloederige) diarree. De oorzaken van beide aandoeningen zijn nog grotendeels onbekend, maar worden in belangrijke mate bepaald door een "westerse" levensstijl. Met name roken heeft een grote invloed op de ernst van beide ziekten. Een belangrijke component in het ontstaan van IBD is een overmatige reactie van het immuunsysteem tegen de, natuurlijk aanwezige, darmflora.
Zowel CD als UC kan nog niet genezen worden, maar wel met behulp van ontstekingsremmende geneesmiddelen als 5-aminosalicylzuur en corticosteroïden tot zekere hoogte onder controle gehouden worden.
Naast de grote invloed van omgevingsfactoren is het voor zowel CD als UC duidelijk dat ook genetische factoren een belangrijke rol spelen. De afgelopen jaren zijn de genetische gegevens van zeer grote groepen CD en UC patiënten en gezonde vrijwilligers geanalyseerd in zogenaamde "genome wide association studies". Deze studies hebben aangetoond dat mutaties in verschillende genen geassocieerd zijn met één dan wel beide aandoeningen.

7.2.1 De lever

De lever is een van de grootste organen in het menselijk lichaam en is in belangrijke mate betrokken bij het "zuiveren" van het bloed.
De lever is opgebouwd uit verschillende typen cellen, waarvan de hepatocyten het grootste deel uitmaakt. Deze cellen zijn de functionele levercellen waarin schadelijke stoffen onschadelijk gemaakt worden. Daarnaast produceren hepatocyten galzouten die essentieel zijn bij de opname van bijvoorbeeld vetoplosbare vitaminen (A, D, E en K) uit de voeding. Hepatocyten liggen in strengen in de lever, omsloten door een bloedvat en een (kleine) galgang. De kleine galgangen komen samen in een grote galgang en deze mondt uit in de galblaas.
Een belangrijke eigenschap van de lever is het vermogen om te regenereren. Dit wil zeggen dat, wanneer levercellen verloren zijn gegaan door ziekte, alcohol- of drugsmisbruik of bijvoorbeeld een operatie, er nieuwe levercellen aangemaakt

worden om de oude te vervangen. Dit proces is zo efficiënt dat in mensen, na het verlies van 50% van de lever massa, de lever binnen enkele maanden weer zijn oorspronkelijke grootte bereikt heeft. In ratten en muizen is na verwijdering van 70% van de lever deze zelfs na 7 tot 10 dagen al weer volledig hersteld.

7.2.2. Leveraandoeningen

Leverziekten komen veel voor en kunnen verschillende oorzaken hebben zoals virale infecties, auto-immuun afwijkingen of geneesmiddel- of alcohol misbruik. Leverziekten worden onderverdeeld in acute en chronische vormen, waarbij acute leverziekten zoals virale infecties of geneesmiddel intoxicatie leiden tot een snelle afname van gezond leverweefsel en medische behandeling nodig is om leverfalen te voorkomen. Chronische leverziekten ontstaan over periodes van jaren door aanhoudende infectie of auto-immuun afwijkingen. Door overactieve "stellaatcellen" wordt een rigide extracellulaire matrix geproduceerd (fibrose) die langzaam delen van de lever inkapselt en bloed en galkanalen afsluit (cirrose). Cirrose kan uiteindelijk leiden tot leverfalen waarna de enige behandeling bestaat uit levertransplantatie.

7.2.3 Leverregeneratie

De lever bezit de bijzondere eigenschap dat deze zich herstelt na schade. Het proces is relatief snel, want na operatieve verwijdering van 70% van de lever in muizen is dit orgaan na 7-10 dagen volledig hersteld. Leverregeneratie is voor een groot gedeelte afhankelijk van deling en vermenigvuldiging (proliferatie) van hepatocyten. Daarnaast zijn er in de lever zones met specifieke leverstamcellen (progenitor cellen). Ook deze cellen vermenigvuldigen zich en kunnen zich gedurende dit proces specialiseren tot functionele levercellen (onder andere hepatocyten). Tot slot zijn ook stamcellen uit het beenmerg betrokken. Deze worden via het bloed in de lever "afgezet" waarna zij zich als de leverstamcellen ontwikkelen tot functionele levercellen. In de meeste leverziekten is de hepatocytproliferatie deels geblokkeerd waardoor activatie van de progenitor cellen een belangrijke rol speelt in het proces van leverregeneratie.

7.3 ATP Binding Cassette (ABC) transporters

De familie van ATP-bindingscassette (ABC) transporteiwitten is een grote familie van eiwitten, verantwoordelijk voor transport van stoffen de cel in en/of uit. De familie bestaat uit 49 leden, welke op basis van structuur en functie zijn onderverdeeld in 7 subfamilies (A t/m G). Deze eiwitten zitten voornamelijk in de celmembraan waardoor ze stoffen van binnen naar buiten en vice versa kunnen verplaatsen. De overeenkomst tussen deze eiwitten is dat ze allemaal energie in de vorm van adenosine

trifosfaat (ATP) gebruiken om het substraat actief te verplaatsen. In het verleden is aangetoond dat enkele van deze ABC-transporter subfamilies in verhoogde mate betrokken zijn bij het veroorzaken van geneesmiddelresistentie van kankercellen. De best beschreven voorbeelden hiervan zijn het Multidrug Resistance protein 1 (MDR1/ABCB1) en het Multidrug Resistance-associated Protein 1 (MRP1/ABCC1). Beide eiwitten transporteren de, voor de kankercel schadelijke, geneesmiddelen de cel uit voor ze het gewenste effect kunnen uitoefenen. Uit voorgaande onderzoeken is gebleken dat de expressie van verschillende ABC transporters verhoogd is in het leverstamcelcompartiment van ratten die onderworpen zijn aan modellen van leverziekten. Tevens vertonen de levers van mensen met verschillende ernstige leverziekten hetzelfde beeld.
Het onderzoek in dit proefschrift richt zich met name op de MRPs en MRP1 in het specifiek.

7.3.1 MRP1

Hoewel sinds de ontdekking van MRP1 in 1992 veel gegevens verzameld zijn over het type geneesmiddelen dat MRP1 kan transporteren en in welke soorten kankercellen MRP1 een rol kan spelen in geneesmiddelresistentie, is nog relatief weinig onderzoek verricht naar de functie van MRP1 in zijn natuurlijke omgeving.
Enkele belangrijke natuurlijke substraten zijn geïdentificeerd welke een tip van de sluier oplichten over de rol van MRP1. De belangrijkste substraten, voor zover bekend, zijn glutathion, een natuurlijke antioxidant; leukotriëen C4, een stof betrokken bij de immuunrespons en stoffen gebonden aan glutathion, sulfaat of glucuronide. Deze laatste groep stoffen ontstaat wanneer, veelal giftige, stoffen in een cel worden omgezet zodat ze beter uit te scheiden zijn via de urine of via de gal. Ook hieruit blijkt dat MRP1 een beschermende rol heeft tegen giftige stoffen. Tevens kan het transport van glutathion en leukotriëen C4 duiden op een functie tijdens ontsteking.

7.3.2 MRP1 in de darmen

Onder normale omstandigheden is MRP1 slechts in kleine hoeveelheden aanwezig in het darmweefsel.
Uit het onderzoek, beschreven in hoofdstuk 2, is gebleken dat in ontstoken darmweefsel van patiënten met CD of UC, de MRP1 expressie verhoogd is. Nader onderzoek heeft uitgewezen dat MRP1 aanwezig is in het epitheel van de darm mucosa en met name in een regio waar ook de darmstamcellen zich bevinden. Om uit te vinden wat de functie van MRP1 is tijdens dit ontstekingsproces is een cellijn van dikke darm epitheelcellen blootgesteld aan een mix van cytokines, die vrijkomen

tijdens ontsteking, al dan niet in combinatie met het remmen of tot overexpressie brengen van MRP1. Bij het remmen van MRP1 werden deze darmcellen gevoeliger voor celdood, terwijl het verhoogd tot expressie brengen van MRP1 de cellen beschermt tegen celdood. Hieruit blijkt dat MRP1 epitheelcellen beschermd tegen de schadelijke werking van deze cytokines.
De verhoogde expressie van MRP1 in stamcellen van de darm tijdens ontsteking lijkt daarmee een natuurlijke reactie te zijn om het darmepitheel te beschermen.
Tevens bevatten immuuncellen (T-lymfocyten/T-cellen) in de darm van patiënten met UC of CD veel MRP1. Om uit te vinden of MRP1 een vergelijkbare functie heeft in T cellen als in epitheel cellen, zijn beide soorten cellen wederom blootgesteld aan cytokines. Uit dit vergelijkende onderzoek (beschreven in hoofdstuk 3) kwam naar voren dat MRP1 een tegengesteld effect heeft in T-cellen en epitheelcellen. Het remmen van MRP1 functie leidt tot minder celdood door cytokines in T-cellen. Wellicht is MRP1 daarom betrokken bij het in goede banen leiden van het ontstekingsproces. Epitheelcellen worden beschermd, terwijl de "schadelijke" T-cellen op den duur versneld opgeruimd worden.

7.3.3 MRP1 in de lever

Om alle functies van de lever goed en efficiënt te laten verlopen, moeten vele stoffen de levercellen in en uit getransporteerd worden. Dit transport wordt onder andere verzorgd door een groot aantal ABC transporters. MRP1 is aanwezig in verschillende celtypen van de lever welke allemaal verschillende functies vervullen.

7.3.1 MRP1 tijdens leverregeneratie

Uit eerder onderzoek naar leverregeneratie is aangetoont dat MRP1 expressie verhoogd is kort na het begin van dit proces. Dit effectis waargenomen in ratten na het uitvoeren van een proefdiermodel voor leverschade.. Opvallend is dat deze verhoogde expressie van MRP1 voornamelijk waar te nemen was in de stamcellen van de lever (progenitor cellen). Een vergelijkbare waarneming werd gedaan in de levers van menselijke patiënten met enkele ernstige (chronische) leveraandoeningen zoals hepatitis C infectie en primaire billiaire cirrose. Ook hier werd meer MRP1 gevonden in de stamcellen van de lever vergeleken met gezond leverweefsel.
Om uit te vinden welke rol MRP1 speelt in het proces van leverregeneratie is in het onderzoek, beschreven in dit proefschrift, een vergelijkbare dierstudie uitgevoerd met muizen welke geen Mrp1 tot expressie brengen (Mrp1 knock-out of Mrp1-/-). In dit onderzoek is leverregeneratie in "normale" muizen vergeleken met leverregeneratie in Mrp1-/- muizen. Er zijn twee modellen van leverregeneratie toegepast. Het

"gewone" model, waarbij ongeveer 70% van de lever verwijderd wordt en een model waarbij stamcel activatie wordt gestimuleerd. Hierbij zijn de muizen eerst behandeld met een stof (2-AAF) die het vermenigvuldigen van hepatocyten blokkeert. Het tweede model is beter geschikt als model voor patiënten met leverziekten omdat het complexe geheel beter wordt nagebootst..

Om te compenseren voor de afwezigheid van Mrp1 in de gezonde lever is de expressie van andere Mrp eiwitten verhoogd. Met name Multidrug resistance associated protein 3 (Mrp3/Abcc3), welke qua structuur het meeste lijkt op Mrp1, lijkt deze rol op zich te nemen. Het levergewicht van zowel de normale (wild type) muizen als de Mrp1 knock-out muizen verschilt niet. Slechts 3 dagen nadat 65% van de levers verwijderd is, is de lever geregenereerd tot 85% van het oorspronkelijke gewicht voor de operatie. In het diermodel voor stamcelactivatie tijdens leverregeneratie is eveneens geen verschil te zien in levergewicht tussen de wild type en de Mrp1 knock-out muizen, zowel voor als 3 dagen na de operatie. Het lijkt er daarom op dat Mrp1 niet essentieel is voor leverregeneratie. Een belangrijke waarneming is dat de expressie van een merker voor stamcelactivatie niet verhoogd is in de levers Mrp1-/- muizen, terwijl dit wel het geval is in de wild type muizen. Dit wijst erop dat de stamcellen in Mrp1-/- muizen anders reageren dan deze in wild type muizen. Om de exacte oorzaak en de eventuele gevolgen hiervan te bepalen zal verder onderzoek gedaan moeten worden. Deze studie is beschreven in hoofdstuk 4.

7.4 Regulatie van MRP1 expressie

Om therapeutisch gebruik te kunnen maken van de beschermende functie van MRP1 in bijvoorbeeld epitheelcellen is het belangrijk te weten hoe MRP1 expressie gereguleerd wordt. Hoewel vele studies de verhoogde expressie van MRP1 beschrijven, gaat het hierbij met name over de geneesmiddel resistente kankercellen. De regulatie van expressie in de "normale" situatie is tot op heden onbekend.

Recent is echter aangetoond dat de MRP1 expressie in de darmen van muizen verlaagd kan worden door PPAR-alpha, een nucleaire hormoon receptor betrokken bij het vetzuurmetabolisme. In hoofdstuk 5 hebben we onderzocht of dit mechanisme ook werkzaam is in menselijke darm en/of levercellen. Activatie van PPAR-alpha in zowel darm- als levercellen heeft geen effect op de expressie van MRP1, zowel op RNA als op eiwitniveau. Op basis van deze resultaten kunnen we concluderen dat MRP1 in de humane lever en darm niet via dezelfde route gereguleerd wordt. Dit betekent dat PPAR-alpha niet gebruikt kan worden om MRP1 expressie in mensen te beïnvloeden.

7.5 Algemene conclusie en toekomstperspectieven

Het in dit proefschrift beschreven onderzoek toont aan dat MRP1 een belangrijke functie heeft in de regulatie van celdood tijdens, of ten gevolge van, ontsteking. Epitheelcellen worden door MRP1 beschermd tegen cytokine geïnduceerde celdood, terwijl T-lymphocyten bij verhoogde MRP1 functie hiervoor juist gevoeliger worden. Hiermee wordt bevestigd dat de functie MRP1 niet beperkt is tot geneesmiddel-resistentie in kankercellen, maar dat dit eiwit ook een belangrijke natuurlijke functie heeft in de bescherming van darmepitheelcellen en de regulatie van normale immuunreactie.

In dit proefschrift hebben wij een beschermende functie van MRP1 laten zien in lever- en darmepitheel. Aanvullend onderzoek is echter noodzakelijk naar de mogelijke klinische toepassing van MRP1 regulatie in bijvoorbeeld de behandeling van "ontstekingsziekten" zoals de ziekte van Crohn en colitis ulcerosa. Tevens zou MRP1 regulatie gebruikt kunnen worden in het verhogen van de efficiëntie van regeneratieve processen zoals leverregeneratie na transplantatie of het verwijderen van tumoren. Gezien de relatief hoge expressie van MRP1 in stamcellen, is ook het gebruik van MRP1 regulatie in stamcelkweek en –therapie niet ondenkbaar.

De moleculaire mechanismen van MRP1 regulatie zijn nog grotendeels onbekend. Dit vormt één van de belangrijkste hordes die genomen moet worden voordat er klinische toepassingen voor MRP1 regulatie mogelijk zullen worden.

Gezien de associatie van MRP1 expressie met geneesmiddelresistentie is het daarnaast van het grootste belang dat uitgesloten wordt dat verhoogde expressie van MRP1 om therapeutische toepassingen leidt tot negatieve bijwerkingen.

Dankwoord

4 jaar onderzoek zijn omgevlogen en liggen momenteel zelfs alweer 2 jaar achter me. Mijn tijd op het lab MDL/kindergeneeskunde was leerzaam en bovenal erg plezierig. Ik mag mij gelukkig prijzen dat ik in september 2004 terecht ben gekomen in een geweldige groep collega's. Ik wil graag van deze gelegenheid gebruik maken om alle mensen te bedanken die hebben bijgedragen aan deze leuke periode en het tot stand komen van dit proefschrift. Een paar wil ik in het bijzonder even noemen.

Beste Klaas Nico, als mijn directe begeleider heb je regelmatig met me om de tafel gezeten om verklaringen te zoeken voor onverwachte en soms ogenschijnlijk onverklaarbare resultaten. Enkele hiervan blijven tot op de dag van vandaag onverklaarbaar, maar hierdoor juist bijzonder interessant. Ik heb bewondering voor je altijd kritische benadering van problemen of resultaten, Daarnaast beheers je de kunst van het schrijven van korte samenvatting tot in de puntjes; geen woord teveel of te weinig.

Beste Han, bedankt voor je vrolijke inbreng in het lab MDL. Naast je wetenschappelijke kennis op het gebied van de lever en celdood heb je mij regelmatig verbaast met je kennis van de meest vreemde sport- en showbizznieuwtjes. Wij zullen je waarschijnlijk nog regelmatig terugzien op tv in een bekende kennisquiz.

Beste Gerard, vrij snel na mijn aanstelling als AIO ben jij betrokken geraakt bij mijn onderzoek. En je hebt een blijvende indruk achter gelaten. De dinsdagmiddag besprekingen waren al snel een begrip geworden op het lab. Tijdens de brainstorm sessies kwamen steeds de wildste ideeën bij je naar boven. Jij ziet geen obstakels, alleen kansen. Je hebt het "out-of-the-box" denken voor mij een gezicht gegeven.

Beste Mariska, mijn steun en toeverlaat op het lab. Ik heb met ontzettend veel plezier met je samengewerkt gedurende vier jaar. Bedankt voor je inzet, ook (en misschien wel juist) toen het onderzoek even niet zo lekker liep. Voor mij ben jij het schoolvoorbeeld van groningse nuchterheid (nait souzen) en ik ben erg blij dat jij als paranimf wilt optreden tijdens mijn promotie. De volgende AIO die met je mag samenwerken mag in zijn of haar handen knijpen.

Uiteraard zijn er meer mensen die ik wil bedanken. Zoals al eerder gezegd, heb

ik kunnen genieten van een geweldige groep collega's. Turbo-tweeling Hans en Tjasso, Lisette, Martijn, Kryszstof, Janette, Fiona, Laura, Antonella, Sandra, Rebecca, Manon, Atta, Golnar en Elise, bedankt voor de gezellige tijd, zowel op het lab als in de kroeg. Ook de "buren" van kindergeneeskunde hebben een grote bijdrage gehad in de goede werksfeer en graag wil ik ook hen hiervoor allemaal bedanken.

Titia & Jannes, naast collega's ook vrienden. Bedankt voor de leuke avonden in "der Witz", aan de Florakade, de Westersingel, in Nijmegen en Malden.

Naast het uitvoeren van onderzoek heb ik bij MDL een aantal studenten mogen begeleiden tijdens hun afstudeerstages. Hier heb ik persoonlijk ontzettend veel plezier aan beleefd en wil Edmee, Marieke, Mark, Moniek en Jessica bedanken voor hun inzet. Ik hoop dat jullie net zulke goede herinneringen bewaren aan jullie tijd bij MDL als ik.

Michiel, alweer bijna 20 jaar mijn beste vriend. Onze regelmatige afspraken (kopje koffie, appelkoek, kip met prut en/of een avond achter de computer) zijn erg belangrijk voor mij geweest en vormen ook vandaag de dag een welkome onderbreking van de dagelijkse hectiek. Ik ben blij dat ook jij mij bij wilt staan als paranimf.

Lieve papa en mama, jullie bijdrage in het voltooien van dit proefschrift is met geen pen te beschrijven. Bedankt voor het vertrouwen dat jullie altijd in mij hebben gehad. Zonder jullie steun was het nooit zover gekomen.

Lieve Eefje, voor mij staat het afronden van dit proefschrift symbool voor het afsluiten van een hoofdstuk in ons leven. Ik heb alleen maar goede herinneringen aan onze periode in Groningen, waar wij in 2004 zijn gaan samenwonen en we ons leven samen zijn gestart. Ik wil je bedanken voor de steun die je me hebt gegeven en ben waanzinnig trots op je dat je "jouw" stad hebt verlaten nadat ik voor een andere richting had gekozen. Mij is de afgelopen jaren heel duidelijk geworden dat ons thuis is waar we samen zijn. Iets dat we gelukkig hebben bezegeld met ons huwelijk afgelopen oktober. Ik hou van je.

Axel

Curriculum Vitae

Axel van Steenpaal werd geboren op 2 maart 1979 in Emmen. Hier voltooide hij in 1997 het VWO aan het Esdal college. In datzelfde jaar begon hij aan de studie Farmacie aan de Rijksuniversiteit Groningen. Zijn afstudeerstage volgde hij binnen de sectie celbiologie van de vakgroep Farmaceutische biologie. Hier voerde hij onderzoek uit naar de vorming van disulfidebruggen in eiwitten na transport door de exportmachinerie van *Bacillus subtilis*. In december 2002 voltooide hij zijn doctoraal Farmacie. Direct aansluitend volgde hij de apothekersopleiding aan de Rijksuniversiteit Groningen. Het apothekersexamen werd succesvol afgerond in augustus 2004.
In september 2004 startte Axel met zijn promotieonderzoek binnen de afdeling maag-, darm- en leverziekten van het Universitair Medisch Centrum Groningen naar de fysiologische functie van Multidrug resistance-associated proteins onder begeleiding van Prof.dr. Klaas Nico Faber en Prof.dr. Han Moshage. De resultaten van het onderzoek zijn beschreven in dit proefschrift.
Sinds november 2008 is Axel werkzaam als Qualified Person binnen Intervet International B.V.

List of publications

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